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Superinfection Exclusion in Duck Hepatitis B Virus Infection

by

Kathie-Anne Walters ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of Doctor of Philosophy

in
Virology

Department of Medical Microbiology and Immunology

Edmonton, Alberta

Fall, 2003

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Faculty of Graduate Studies and Research

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Abstract

Chronic infection with hepatitis B virus leads to an increased risk for the development of cirrhosis and hepatocellular carcinoma. Lamivudine is an inhibitor of hepadnavirus replication and is used to treat chronic HBV infections and prevent re-infection of transplanted livers. Lamivudine-resistant HBV variants do arise during prolonged therapy, indicating a need for additional antiviral drugs. The first goal of this thesis was to develop an assay to screen for antiviral-compounds active against the lamivudine-resistant HBV variants. Replication-competent HBV constructs containing the rtL180M/M204V or rtM204I mutations associated with lamivudine resistance were used to produce stable cell lines that express the resistant virus. HBV produced by these cell lines was shown to have a marked decrease in sensitivity to lamivudine, reduced sensitivity to penciclovir but was still inhibited by the nucleoside analogues CDG (carbocyclic 2'-deoxyguanosine) and abacavir.

The second goal of this thesis was to use the duck hepatitis B virus animal model to investigate whether superinfection exclusion occurs in hepadnaviral infections. A genetically-tagged strain of DHBV, DHBV-*ClaI*, was used to distinguish the superinfecting virus from the wild-type virus present in infected animals. Superinfection exclusion was observed both *in vivo*, in DHBV-infected animals, and *in vitro*, using DHBV-infected primary duck hepatocytes.

Exclusion is specific to DHBV as adenovirus, herpes simplex virus and vesicular stomatitis virus are able to infect DHBV-infected hepatocytes. Transduction of primary duck hepatocytes with recombinant adenoviruses expressing various DHBV antigens

indicated that the large surface antigen was independently capable of mediating exclusion.

As the large surface antigen has been shown to down-regulate carboxypeptidase D, a known cellular receptor for DHBV, this was investigated as a possible mechanism of exclusion. Time-course experiments indicate that there is no correlation between DHBV-*ClaI* exclusion and decreases in the levels of carboxypeptidase D in target hepatocytes. In addition, a mutant large surface antigen which does not down-regulate carboxypeptidase D was still capable of inhibiting DHBV infection. In summary, the superinfection exclusion observed in duck hepatitis virus infection is mediated by the large surface antigen. However, the mechanism does not appear to be a reduced ability to bind or enter DHBV-infected cells as a result of a down-regulation of carboxypeptidase D.

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Table of Contents

Chapter 1 : Introduction

1.1 Hepadnaviridae family.........1

1.2 Duck hepatitis B virus.........2

  1.2.1 Virion structure.........2

  1.2.2 Viral genome.........2

1.3 Duck hepatitis B virus life cycle.........4

  1.3.1 Attachment to hepatocytes.........6

  1.3.2 Viral entry into hepatocytes.........15

  1.3.3 Nucleocapsid disassembly and nuclear import of viral DNA.....18

  1.3.4 Formation of covalently closed circular DNA (cccDNA).........19

  1.3.5 Viral Transcription.........20

  1.3.6 Translation of viral gene products.........25

  1.3.7 Viral RNA encapsidation and assembly of pre-initiation complex.........31

  1.3.8 Assembly of nucleocapsids.........34

  1.3.9 Overview of DNA replication.........34

  1.3.10 Priming of reverse transcription and (-) DNA strand synthesis...36

  1.3.11 Synthesis of (+)DNA strand.........39

  1.3.12 Virus morphogenesis.........41

1.4 Viral gene products.........42

  1.4.1 Precore protein (antigen).........43

  1.4.2 Core protein.........43

  1.4.3 Polymerase protein.........47

  1.4.4 L and S surface antigen.........51

1.5 References.........60

Chapter 2 : Generation of Stable Lamivudine-Resistant Hepatitis B Virus Cell Lines For Antiviral-Compound Screening

2.1 Introduction.........79

2.2 Materials and Methods.........83

2.2.1 Plasmids and mutagenesis 83
2.2.2 Cell culture, transfection and clone selection.....86
2.2.3 Characterisation of individual clones.....87
2.2.4 Analysis of intracellular viral DNA.....87
2.2.5 Integration of HBV DNA.....88
2.2.6 Antiviral compounds.....89
2.2.7 Drug assay.....89
2.3 Results.....90
2.3.1 Generation and characterisation of stable HBV-producing clones.....90
2.3.2 Analysis of antiviral activity.....95
2.4 Discussion.....99
2.5 References.....104

Chapter 3 : Superinfection Exclusion in Duck Hepatitis B Virus Infection.

3.1 Introduction.....112
3.2 Materials and Methods.....114
3.2.1 Animals and virus stocks.....114
3.2.2 Extraction of extracellular viral (ECV) DNA from serum.....114
3.2.3 Preparation and infection of primary duck hepatocytes.....114
3.2.4 Isolation of intracellular viral DNA from primary duck hepatocytes.....115
3.2.5 Analysis of viral DNA.....115
3.2.6 Cell sorting and single-cell PCR.....116
3.3 Results.....118
3.3.1 DHBV-*ClaI* establishes an infection in uninfected but not congenitally infected animals.....118
3.3.2 Co-infection of ducklings with DHBV and DHBV-*ClaI*.....121
3.3.3 Staggered co-infection of ducklings with DHBV and DHBV-*ClaI*.....122
3.3.4 A lamivudine-resistant virus, DHBV-M512V, is unable to

efficiently establish an infection in congenitally-infected ducks despite having a selective advantage.............................125
3.3.5 Exclusion of DHBV-*ClaI* in PDH cultures.......................127
3.4 Discussion...133
3.5 References...138

Chapter 4: Mechanism of Duck Hepatitis B Virus Superinfection Exclusion

4.1 Introduction...142
4.2 Materials and Methods..144
4.2.1 Cells, virus stocks and infections................................144
4.2.2 Generation of recombinant adenovirus.............................145
4.2.3 Fluorescent labelling of DHBV....................................149
4.2.4 Binding studies and confocal microscopy..........................149
4.2.5 Infection of PDHs with recombinant adenovirus....................149
4.2.6 SDS-PAGE and Western blot analysis of DHBV antigens and carboxypeptidase D 150
4.3 Results..151
4.3.1 Uninfected and DHBV-infected PDHs are equally susceptible to infection with adenovirus, VSV, and HSV..........................151
4.3.2 DHBV gene expression is required for exclusion of DHBV-*ClaI*..153
4.3.3 The identification of the DHBV antigen responsible for superinfection exclusion...155
4.3.4 Potential mechanism of L surface antigen-mediated exclusion of DHBV infection..162
4.3.5 Binding of rhodamine-labelled virus to uninfected and DHBV-infected PDH...164
4.3.6 Confocal microscopy analysis of uninfected and DHBV-infected PDHs incubated with rhodamine-labelled DHBV......................164
4.3 Discussion...169
4.4 References...174

Chapter 5: Summary and Future Considerations

5.1 Summary 177
5.2 Directions for future work 179
5.3 Significance of superinfection exclusion in viral infections 180
5.4 References 183
Appendix I: Plasmid maps 187

List of Figures

Figure 1.1 Virion structure and genome organisation....3

Figure 1.2 Duck hepatitis B virus life cycle....5

Figure 1.3 Schematic of L surface antigen functional domains....7

Figure 1.4 Relationship between DHBV genome, ORFs and mRNA transcripts...22

Figure 1.5 Schematic of DHBV genome replication....35

Figure 1.6 Proposed models of L surface antigen topology....56

Figure 2.1 Chemical structures of nucleoside analogues....80

Figure 2.2 Map of pCMV-HBV-neo construct used to generate stable cell lines rtM204V and rtL180M/M204V....84

Figure 2.3 Production of intracellular viral replicative intermediates in 2.2.15, rtM204V and rtL180M/M204V cell lines....93

Figure 2.4 Integration of HBV sequences into genomic DNA of HepG2, rtM204V and rtL180M/M204V cell lines....94

Figure 2.5 IC_{50} graphs for lamivudine, penciclovir and abacavir using the rtL180M/M204V cell line....97

Figure 3.1 Partial nucleotide sequences of wild-type DHBV, DHBV-*ClaI* and DHBV-M512V....117

Figure 3.2 Sensitivity of the PCR assay used to distinguish DHBV from DHBV-*ClaI*....119

Figure 3.3 Infection of naïve and congenitally-infected ducklings with DHBV-*ClaI*....120

Figure 3.4 Sorting of primary duck hepatocytes into single-cells....123

Figure 3.5 Single-cell PCR analysis of viral population in DHBV-infected hepatocytes....124

Figure 3.6 Staggered co-infection of naïve ducklings....126

Figure 3.7 Serum viral DNA analysis of congenitally-infected ducklings treated with lamivudine and infected with DHBV-M512V....128

Figure 3.8 Exclusion of DHBV-*ClaI* in primary duck hepatocytes....................130

Figure 3.9 Southern analysis of intracellular virus from DHBV-infected primary duck hepatocytes..132

Figure 4.1 Schematic outline of the AdEasy system..................................146

Figure 4.2 Superinfection exclusion is limited to DHBV.............................152

Figure 4.3 Superinfection exclusion of DHBV-*ClaI* is dependent on antigen expression during the initial DHBV infection............................ 154

Figure 4.4 Expression of DHBV antigens from recombinant adenoviruses...... 156

Figure 4.5 Superinfection exclusion is mediated by L surface antigen (Part I)....158

Figure 4.6 Western blot analysis of adenovirus-infected PDH........................159

Figure 4.7 Superinfection exclusion is mediated by L surface antigen (Part II)...160

Figure 4.8 Superinfection exclusion is mediated by L surface antigen (Part III)...161

Figure 4.9 Superinfection exclusion of DHBV-*ClaI* does not correlate with a decrease in carboxypeptidase D expression...............................163

Figure 4.10 Binding of rhodamine-labelled DHBV to uninfected PDH............165

Figure 4.11 Binding of rhodamine-labelled DHBV to DHBV-infected PDH......166

Figure 4.12 Confocal microscopic analysis of uninfected and DHBV-infected PDH incubated with rhodamine-labelled DHBV....................................168

List of Tables

Table 2.1 Production of HBsAg, HBeAg and extracellular viral DNA..........91

Table 2.2 Effect of nucleoside analogues on wild-type and lamivudine-resistant HBV..........96

Table 3.1 Inoculation of congenitally-infected and uninfected ducklings with lamivudine-resistant DHBV-M512V..........129

Abbreviations and Definitions

ATP	adenosine triphosphate
bp	base-pair
C	cytidine
cccDNA	covalently closed circular DNA
CDG	carbocyclic 2'-deoxyguanosine
CD4	cluster designation 4
CGD	chicken glycine decarboxylase
CPD	carboxypeptidase D
CPE	cytopathic effect
° C	degrees celcius
dCTP	deoxycytidine triphosphate
DGD	duck glycine decarboxylase
dGMP	deoxyguanosine monophosphate
dGTP	deoxyguanosine triphosphate
DHBV	duck hepatitis B virus
DMEM	Dulbucco's modified essential media
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DR1	direct repeat 1
DR2	direct repeat 2
ECV	extracellular virus
EDTA	ethylene-diaminetetra-acetic acid
ER	endoplasmic reticulum
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
gp	glycoprotein
GSHV	ground squirrel hepatitis B virus
GST	glutathione-S-transferase

GTPase	guanosine triphosphatase
HBeAg	hepatitis B virus e antigen
HBsAg	hepatitis B virus surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HHBV	heron hepatitis B virus
HIV	human immunodeficiency virus
HNF	hepatocyte nuclear factor
HRP	horse-radish peroxidase
Hsp90	heat shock protein 90
HSV-1	herpes simplex virus-1
IC_{50}	50% inhibitory concentration
IFN	interferon
i.m.	intra-muscular
kb	kilobase
kbp	kilobase pair
kDa	kilodalton
LB	Luria Broth
L-FMAU	L-2’-deoxy-2’-fluor-5-methyl-1-ß-D-arabinosyluracil
LsAg	large surface antigen
MEM	minimal essential media
$MgCl_2$	magnesium chloride
ml	millilitres
mRNA	messenger RNA
MOI	multiplicity of infection
MoMLV	Moloney murine leukemia virus
NaCl	sodium chloride
NLS	nuclear localisation signal
nm	nanometer
nM	nanomolar

nt	nucleotide
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDH	primary duck hepatocytes
PEG	polyethylene glycol
PFA	phosphonoformic acid
pgRNA	pregenomic RNA
pol	polymerase
Ppi	pyrophosphate
PRE	post-transcriptional regulatory element
PTB	polypyrimidine binding protein
RBP-1	Ran binding protein 1
rc	relaxed-circular
RNA	ribonucleic acid
RSV	Rous sarcoma virus
rt	reverse transcriptase
SAPK	stress activated protein kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SGHV	snow goose hepatitis B virus
SPRK-1 and 2	serine protein-specific kinase 1 and 2
S sAg	small surface antigen
SVP	subviral particle
SV40	simian virus 40
TBE	Tris-boric acid-EDTA
TM1 and 2	transmembrane domain 1 and 2
tRNA	transfer RNA
U	uridine
UBS	upstream binding protein
ug	microgram

ul	microlitre
uM	micromolar
UV	ultraviolet
VGE	viral genome equivalent
VSV	vesicular stomatitis virus
WHV	woodchuck hepatitis B virus
wt	wild-type

Chapter 1

Introduction

1.1 Hepadnaviridae family.

Hepadnaviridae are a group of small, enveloped DNA viruses with marked hepatotropism (1). The viruses in this family share many characteristics (1). First, the hepadnavirus genome is a small (3-3.3 kb), partially double-stranded DNA genome which is organised into three (avian) or four (mammalian) overlapping reading frames encoding the viral proteins. Second, these viruses employ a unique replication strategy where the viral DNA is replicated via reverse transcription of an RNA intermediate. Third, infection is characterised by strict species and hepatic tropism. Fourth, these viruses have the ability to establish chronic infections in the absence of direct cellular cytopathicity. Fifth, a hallmark of hepadnaviral infections is the production of an excess amount of surface antigen-containing particles from infected hepatocytes which are released into the serum of infected individuals.

The family is subdivided into two groups: mammalian hepadnaviruses include human hepatitis B virus (HBV), which is the prototypic member of the family, ground squirrel hepatitis B virus (GSHV) and woodchuck hepatitis B virus (WHV); avihepadnaviruses include DHBV, grey heron hepatitis B virus (HHBV) and snow goose hepatitis B virus (SGHV) (1). Because of the species-specificity of hepadnaviruses, a direct animal model to study the complete HBV life cycle is limited to the expensive chimpanzee model. However, other members of the family provide good animal models for the study of hepadnaviruses. For example, the WHV animal model has been extremely useful in studying HBV-related hepatocellular carcinoma (HCC) as a large percentage of chronically-infected animals develop HCC. The DHBV animal model has proven to be invaluble in the study of hepadnaviruses, in part because it provides the only efficient *in vitro* infection system. Chronic DHBV infection in ducks is not associated with any pathology. While this makes it an unsuitable model for studying hepadnavirus-mediated liver disease, it is still practical for studying both antivirals and various aspects of the virus life cycle.

1.2 Duck hepatitis B virus.

1.2.1 Virion structure.

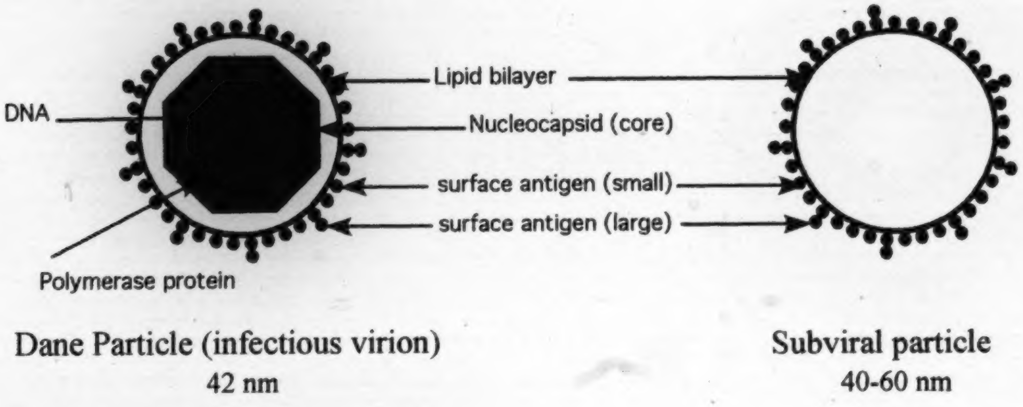
Two types of viral particles (depicted in Figure 1.1a) are produced during DHBV infection. The Dane particle is 42-47 nm in diameter and is the infectious virion (1). The outer shell of the virion is composed of a lipoprotein envelope, derived from the endoplasmic reticulum (ER) membrane of the infected cell, containing the viral-encoded small and large surface antigens. The inner core is comprised of 180 subunits of the core protein, a 31 kDa phosphoprotein, which assembles into an icosahedral nucleocapsid. Within this core is the circular, partially double -stranded viral DNA which is covalently attached to the polymerase protein at the 5' end of the (-) strand DNA. The other viral particle produced is the sub-viral particle (SVP) which is a 40-60 nm sphere produced in 100-1000 fold excess of the Dane particles. SVPs are composed entirely of surface antigen and host-derived lipid. They do not contain viral DNA and so these particles are non-infectious. In HBV infection, an additional SVP in the shape of filaments is produced.

1.2.2 Viral genome.

The hepadnaviral genome is a circular, partially duplex DNA approximately 3 kb in length (1). As shown in Figure 1.1b, the two strands are not identical. The (-) strand DNA is genome-length with the polymerase protein covalently bound to its 5' terminus. The (+) strand DNA is less than genome length, which results in a single-stranded segment of variable length, and has a capped RNA at its 5' terminus. The 5' ends of both strands map to short direct repeats, DR1 and DR2 for (-) strand and (+) strands, respectively.

The organisation of the DHBV genome, also shown in Figure 1.1b , is remarkably compact. Every nucleotide is within at least one open reading frame (ORF). The largest open reading frame encodes the viral polymerase, which has both RNA-dependent and DNA-dependent DNA polymerase activity as well as RNaseH activity. Partially overlapping the 5' end of the pol ORF is the core ORF which encodes the viral capsid

A. Duck hepatitis B virus particles


DNA
Lipid bilayer
Nucleocapsid (core)
surface antigen (small)
surface antigen (large)
Polymerase protein
Dane Particle (infectious virion)
42 nm
Subviral particle
40-60 nm

B. Genome organization of DHBV

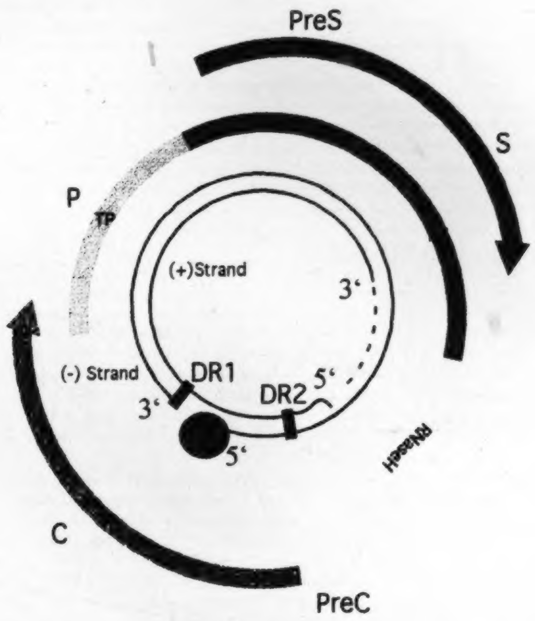

PreS
S
P
TP
(+)Strand
3'
(-) Strand
DR1
DR2
5'
3'
5'
RNaseH
C
PreC

Figure 1.1: (A) Schematic of DHBV particles and (B) genome with corresponding open reading frames. ●: polymerase protein. DR1: direct repeat 1. DR2: direct repeat 2. P: polymerase. PreC: precore. C: core. PreS: pre-surface. S: surface. Solid line: complete viral DNA. Dashed line: incomplete viral DNA.

protein, core, and a secreted form of core termed e antigen. This is accomplished through the use of two in-frame AUG start sites. This mechanism is also used in the surface antigen open reading frame, which encodes the viral envelope proteins. Two in-frame AUG start sites allow the expression of two envelope proteins, large (L) and small (S) surface antigen, from a single open reading frame. The entire surface open reading frame overlaps with the polymerase open reading frame.

1.3 Duck hepatitis B virus life cycle.

The life cycle is similar for all hepadnaviruses and is depicted in Figure 1.2 (1). Infection is initiated by the interaction of the virus with a receptor present on the surface of hepatocytes. The virus then enters the cell, likely by endocytosis, and the nucleocapsid is released into the cytoplasm. Transport of the nucleocapsid to the nuclear membrane is mediated by a nuclear localisation signal (NLS) present on the core protein. Disassembly of the nucleocapsid, either in the cytoplasm or at the nuclear membrane, is followed by release of the viral DNA into the nucleus. The relaxed circular genome is then converted into covalently closed, circular DNA(cccDNA) which serves as the template for virus transcription. The viral genome is transcribed by host RNA polymerase II and the transcripts are transported to the cytoplasm. Following translation of the viral gene products in the cytoplasm, the pregenomic RNA is packaged along with the viral polymerase into nucleocapsids where DNA synthesis occcurs. Reverse transcription of the pregenomic RNA followed by DNA-dependent DNA polymerisation results in the relaxed-circular, partially double-stranded genome. At this point the nucleocapsids are either re-directed to the nucleus or, alternatively, they can bud into the ER lumen and exit the cell throught the secretory pathway as enveloped, infectious virions.

Duck Hepatitis B Virus Life Cycle

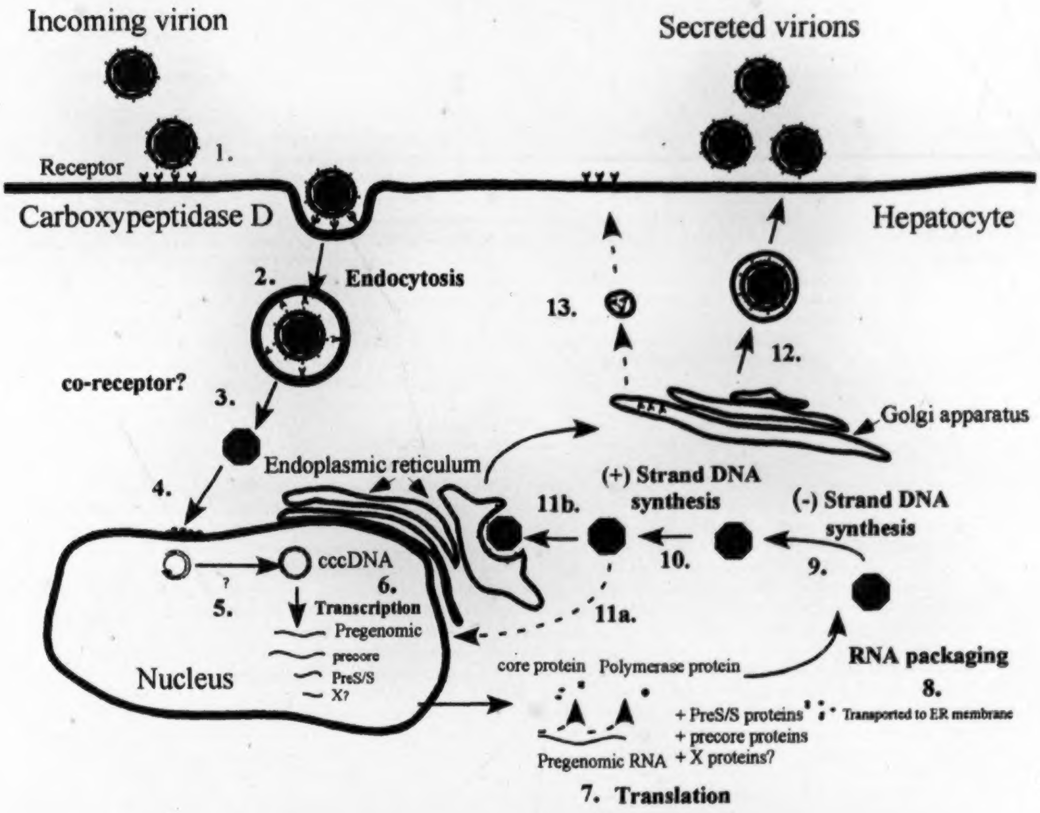

Incoming virion
Secreted virions
Receptor
1.
Carboxypeptidase D
Hepatocyte
2.
Endocytosis
13.
12.
co-receptor?
3.
Golgi apparatus
4.
Endoplasmic reticulum
(+) Strand DNA synthesis
(-) Strand DNA synthesis
11b.
10.
9.
cccDNA
?
5.
6.
Transcription
Pregenomic
precore
PreS/S
X?
11a.
Nucleus
core protein
Polymerase protein
RNA packaging
8.
+ PreS/S proteins
Transported to ER membrane
+ precore proteins
+ X proteins?
Pregenomic RNA
7. Translation

Figure 1.2: Steps of DHBV life cycle: 1. Virion attaches to carboxypeptidase D (ɣ) present on the surface of hepatocyte. **2.** Virion enters the hepatocyte via endocytosis. **3.** Viral/endosomal membranes fuse and the nucleocapsid is released into the cytoplasm. **4.** Nucleocapsid is targeted to the nuclear membrane and viral DNA is released into the nucleus. **5.** The relaxed-circular genome is converted into the covalently closed circular (ccc)DNA. **6.** RNA polymerase II-mediated synthesis of viral RNA transcripts using cccDNA as the template. **7.** Nuclear export of viral RNA transcripts and translation of viral gene products. **8.** Polymerase protein mediated packaging of viral pregenomic RNA and assembly of nucleocapsid. **9.** Synthesis of (-) strand DNA within the nucleocapsid. **10.** Synthesis of (+) strand DNA. **11.** Nucleocapsid containing mature viral DNA is either: **a.** Directed to nucleus where relaxed-circular viral DNA is converted into cccDNA or **b.** directed towards endoplasmic reticulum membrane where the interaction of the large surface antigen with the nucleocapsid facilitates virus envelopement. **12.** Enveloped virus exits cell via secretory pathway. **13.** Re-cycling of carboxypeptidase D between cell surface and Golgi.

1.3.1 Attachment to hepatocytes.

As mentioned previously, the DHBV genome encodes for two envelope proteins, termed L and S surface antigen. S surface antigen is encoded by the surface open reading frame while L surface antigen is comprised of the surface open reading frame plus an additional region, termed PreS, at the amino terminus. It has been established that the L surface antigen, and more specifically the PreS region, is responsible for the attachment of the virus to hepatocytes (78). Initial evidence for the role of the PreS region in attachment came in part from mapping the epitopes of neutralizing monoclonal antibodies. A monoclonal antibody, SD20, with a neutralising activity of 77%, both *in vitro* and *in vivo*, was found to bind to a region between aa 77 and aa 100 in the PreS region (86, 87). This region is highly conserved among different DHBV isolates and coincides with a computer predicted major antigenic site. The same antibody did not react with the PreS proteins of HHBV which varies in sequence from DHBV PreS in this region. Fine mapping of the epitopes of the Mab SD20 as well as Mab900, which has a DHBV neutralising activity of 90%, revealed that Mab SD20 recognised the region between aa 100 and aa 105 whereas Mab900 recognised the region between aa 83 and aa 90 of PreS (23). Polyclonal antiserum directed against the first 131 aa of PreS, which completely abolishes DHBV infectivity in ducklings, recognises a region between aa 82 and aa 95 (23). Additional neutralising epitopes have been identified, including the regions aa 58-66, aa 139-145, aa 91-99 (181) and aa 98-104 of PreS (93). Again, these regions appear to be highly conserved among different strains of DHBV, particularly the regions between aa 91-99 and aa 139-145, despite the overall high sequence variation in the PreS region (181). It is speculated that these three regions represent regions of a conformational site present in the native L surface antigen. The positions of the identified neutralising epitopes present on the L surface antigen are shown in Figure 1.3. Also shown are the regions known to be involved in binding to the cellular receptor carboxypeptidase D (CPD) as well as the potential co-receptor, duck glycine decarboxylase (DGD). Note the overlap in neutralising epitopes and receptor binding domains, with the exception of the neutralising epitope located at aa 139-145.

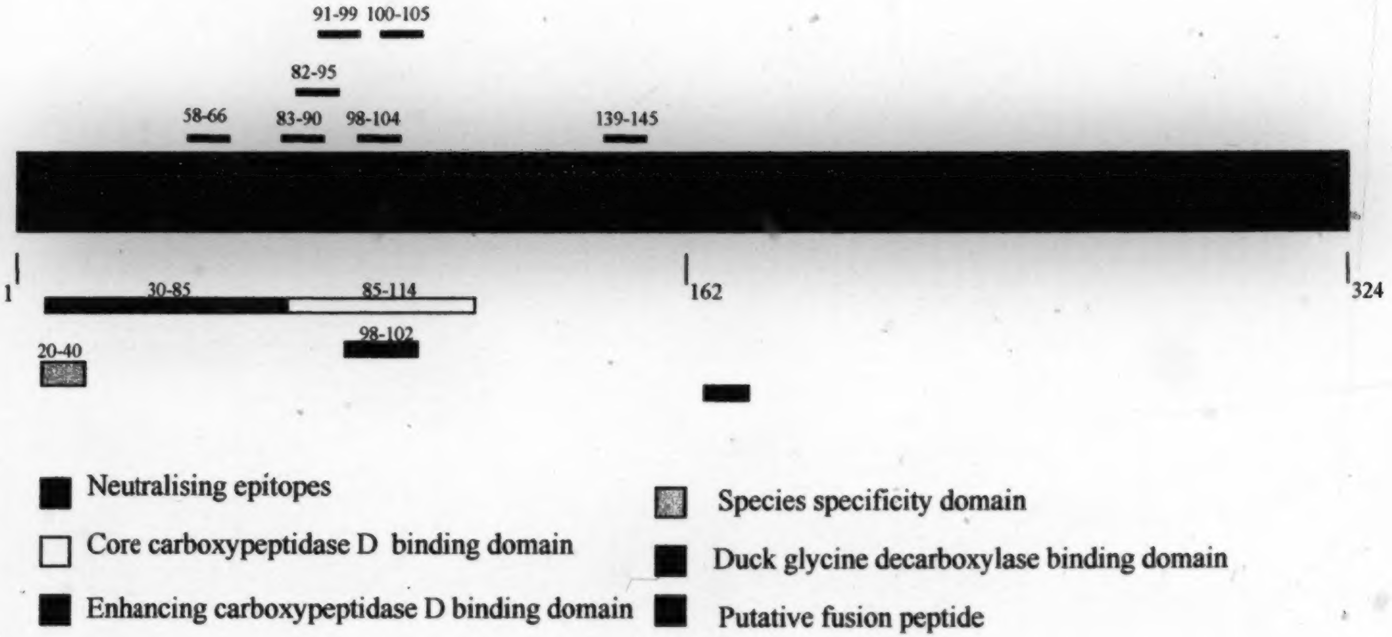

91-99
100-105
82-95
58-66
83-90
98-104
139-145
1
162
324
30-85
85-114
98-102
20-40
■ Neutralising epitopes
☐ Core carboxypeptidase D binding domain
■ Enhancing carboxypeptidase D binding domain
▨ Species specificity domain
■ Duck glycine decarboxylase binding domain
■ Putative fusion peptide

Figure 1.3: Functional segments and positions of neutralising epitopes in DHBV Large surface antigen. Numbers represent amino acid position in large surface antigen.

The minimal binding region of the neutralising Mab900 was mapped to ^{88}WTP^{90} of PreS (23). Analysis of PreS variants selected *in vivo* that escaped neutralisation by Mab900 revealed that the majority of the changes involved point mutations at position ^{90}P as well as an upstream proline residue, ^{5}P (153). These mutants were shown to retain their infectivity *in vivo*, although the majority of the variants containing substitutions at ^{90}P were found to revert to wild-type when no longer under immune pressure by Mab900 (153). Amino acids substitutions in the PreS domain at this position have been shown to substantially reduce Mab900 binding, explaining the loss of Mab900's neutralising activity against these variants (23). Single amino acid substitution indicated that ^{88}W was also important for Mab900 binding to PreS (23). Within the ^{88}WTP^{90} epitope, substitutions of any kind to ^{88}W are least tolerated with regard to MAb900 binding (23).

The role of these PreS regions in the immune response of ducks has also been investigated by mapping the epitopes of antibodies present in the serum of animals that have successfully cleared DHBV infection. Cheung *et al* analysed the antibodies present in convalescent sera using a competitive inhibition assay with epitope-defined monoclonal antibodies against the duck L surface antigen (29). Although the antibodies to the PreS and S domains were found to be highly variable in terms of extent and specificity, antibodies to a previously identified neutralising epitope, aa 91-99 of the PreS domain, were present in all convalescent animals (29). In a similar study, Chassot *et al* identified five major epitopes recognised by antibodies from adult ducks immunized with either DHBV or bacterially-expressed PreS domain (24). One of these regions, aa 58-65, coincided with a previously identified neutralizing epitope, aa 58-66 (181).

Because the majority of the neutralising epitopes map to the PreS region of the L surface antigen, it is logical to assume that it is this viral protein that is responsible for attachment of the virus to the hepatocyte and that the neutralising antibodies disrupt the virus-cell interaction. Indeed, competitive binding studies using primary duck hepatocytes (PDH) and yeast-derived SVPs containing only one of the two DHBV surface antigens revealed that only the L surface antigen was capable of inhibiting both DHBV binding and infection (78). Bacterially-expressed L surface antigen is also capable of inhibiting DHBV infection of PDH (166). Rabbit antibodies against two overlapping peptides, aa 83-97 and aa 93-107, containing three major neutralisation epitopes were found to inhibit binding of DHBV to PDH as well as neutralise virus infectivity *in vitro* (154). Amino acid substitution also revealed a correlation between the PreS residues which are critical for Mab900 neutralisation, ^{88}WTP^{90}, and those that are required for cell interaction. This indicates that the same residues are involved in both DHBV neutralisation and virus attachment (154).

Identification of the cellular receptors for hepadnaviruses has proven to be difficult. This is at least partly due to the lack of an efficient *in vitro* culture system for the majority of the hepadnaviruses. However, at least one receptor has been identified for DHBV, although it appears that additional co-receptor(s) are required for entry of DHBV into cells. Two groups of researchers independently identified a cellular glycoprotein, gp170/180, as a potential receptor for DHBV. Kuroki *et al* isolated a PreS-binding protein by coprecipitating labeled duck hepatocyte lysates with DHBV particles or L surface antigen using monoclonal anti-PreS antibodies (82). Tong *et al* used a glutathione-S- transferase (GST)-PreS fusion protein to isolate a PreS- binding protein from ^{35}S methionine-labeled duck hepatocyte lysates (163). Subsequent protein sequencing and cDNA cloning revealed that both proteins are the same carboxypeptidase-like protein, now called duck carboxypeptidase D (CPD) (83, 163).

CPD, or gp180 as it is also known, belongs to a family of basic carboxypeptidases that remove basic amino acids (lysine or arginine) from the C-termini of proteins (83). It is a type I transmembrane glycoprotein which consists of a large extracellular domain, a hydrophobic transmembrane domain and a short (58 residues) cytoplasmic C-terminal tail (146). The large extracellular region consists of three 50 kDa domains (named A,B,C) with domain A positioned farthest from the plasma membrane and domain C closest to the plasma membrane. The enzymatic activity resides in domains A and B while the function of domain C is unknown. Unlike other viral receptors, CPD is primarily localised to the Golgi apparatus and only transiently cycles to the cell surface (15, 42, 82). The cytoplasmic tail contains sequences which are responsible for targeting to the Golgi apparatus and deletion of this region arrests CPD at the cell surface (15, 42).

There is substantial evidence to support CPD as a receptor for DHBV. First, CPD binds specifically with high affinity to the L surface antigen (82, 83, 163). In fact, one study found it to be the only high affinity L surface antigen-binding protein present in duck hepatocytes (15). The interaction between DHBV L surface antigen and CPD is known to be specific because it can be competitively inhibited by an excess of free L surface antigen or by viral particles (15). In addition, soluble CPD inhibits DHBV infection of PDH, as do anti-CPD antibodies (168, 169). Furthermore, the region of PreS found to be responsible for CPD binding is the same region previously shown to be important for the interaction of DHBV with a cellular receptor (166). This region also corresponds to the previously discussed PreS region that contains several major neutralising epitopes and which is highly conserved among DHBV strains. Indeed, the L surface antigen-CPD interaction was found to be inhibited by neutralising, but not non-neutralising antibodies (82, 83, 163). This indicates that antibody inhibition of DHBV infection is mediated by blocking the L surface antigen-CPD interaction. Mutations in the PreS region that block CPD binding also destroy virus infectivity.

Efficient L surface antigen-CPD interaction appears to be limited to the duck CPD, as DHBV L surface antigen does not efficiently bind the chicken homologue of CPD (15). This suggests that the L surface antigen-CPD interaction might be partially responsible for the species specificity characteristic of hepadnaviruses. However, HHBV L surface antigen was found to efficiently bind duck CPD, despite 50% amino acid difference between duck and heron PreS sequences (15, 166). This suggests that hepadnavirus interaction with the cellular receptor is determined by the 3-D structure of PreS rather than the primary sequence. HHBV does not infect Pekin ducks and can only inefficiently (approximately 1%) infect PDHs (72). Whether this is due to inefficient binding to the hepatocytes or other factors has not been determined. HHBV PreS also interacts with the heron homologue of CPD indicating that CPD may be a common component of the receptor complex for avian hepadnavirus (15).

Second, expression of CPD mediates attachment and internalisation of DHBV into cells that are refractory to DHBV infection (15, 162). DHBV binding to CPD-expressing cells was inhibited by neutralising antibodies which recognize the region of PreS involved in the CPD interaction (162). As well, DHBV binding was not observed when cells were transfected with a truncated version of CPD which does not bind PreS (162). Internalisation of viral particles is inhibited when CPD is arrested at the cell surface by deletion of the cytoplasmic tail (15) or when infections were done at either at 4°C or in the presence of energy-depleting agents (162). These results support the idea that DHBV enters cells by endocytosis rather than by fusion at the cell surface. Although CPD is capable of mediating viral internalisation, it is not sufficient to allow a productive infection (162). An additional species and/or tissue specific co-receptor appears to be required, most likely to mediate fusion between the endosome and viral envelopes to complete virus entry into the cytoplasm following endocytosis. Third, CPD is down-regulated in both DHBV-infected liver or L surface antigen-expressing PDHs (14).

The regions of L surface antigen and CPD that are involved in the interaction have been identified. L surface antigen binds to domain C of CPD, positioning the virus in close proximity to the host cell membrane which is ideal for viral/cell membrane fusion (41, 42, 146, 169). A short region in the "N-terminus" of domain C, comprising amino acids 920-949, is essential for DHBV binding (146). The enzymatic activity of CPD does not appear to be required for L surface antigen binding as deletion of domains A and B, which are the enzymatically active regions, does not affect L surface antigen binding.

The domain of PreS that is involved in the CPD interaction is more extensive, comprising residues 30-115 (15, 73, 163). This is consistent with results obtained from mapping the epitopes of neutralising antibodies which suggested that a conformational epitope spanning a large region was important for the virus-cell interaction. The CPD-binding domain of the PreS region is comprised of a core binding domain, aa 85-115, which is essential for CPD interaction as well as an N-terminal region starting at aa 30 which enchances affinity (15). Site-directed mutagenesis revealed that the PreS residues 95 and 97 are critical for CPD binding (163). Interestingly, these residues are also located near the center of a neutralising epitope.

The conformation of both CPD and PreS domains appear to play an important role in this interaction for several reasons. First, only antibodies generated against native CPD are capable of inhibiting DHBV infection (169). Second, the PreS-CPD interaction is sensitive to internal deletions in this area of PreS (166). Third, HHBV PreS efficiently binds duck CPD despite a 50% aa divergence in the PreS binding domain (166).

Interestingly, most of the receptor binding domain of PreS has been shown to be in a random conformation without tertiary structure (169). It is possible that the potential of PreS to form a particular 3-D structure after the initial contact with CPD, rather than a constant conformation, is critical for the stable interaction. In support of this theory, fluorescence spectra, circular dichroism and two-dimensional NMR show that the formation of a stable ligand-receptor complex induces significant conformational changes in the entire PreS-CPD complex (169).

The L surface antigen-CPD interaction has been extensively studied. Evidence from Urban *et al* suggests that DHBV uses a unique two-step process for its interaction with its receptor (169). There is an initial contact between CPD and the essential binding domain of PreS. This initial low affinity interaction is then stabilized sequentially via sequences N-terminal to the essential binding domain.

There are several lines of evidence that CPD is not the sole receptor used by DHBV to gain entry into hepatocytes. First, CPD is naturally expressed in a wide range of tissues, including many that are not sites of DHBV replication (82, 163). Second, PDHs cultured in the presence of serum quickly de-differentiate and lose the ability to be infected by DHBV (131, 165). The loss of susceptibility to DHBV infection correlates with a decrease in ability of cells to efficiently bind virus particles. However, CPD expression does not appear to decrease as hepatocytes are cultured, although it is possible that CPD cycling to the cell surface decreases (14). In addition, congenitally-infected PDH continue to support virus replication long after cells become resistant to DHBV infection (Walters, personal observation). Together, these results suggest that the block in infection is due to diminished expression of a liver-specific receptor lost during de-differentiation of the cultured hepatocytes. Third, studies using HHBV, which does not infect Pekin ducks, psuedotyped with DHBV/HHBV chimeric surface proteins have shown that host specificity is determined at the level of virus entry and involves aa 22-90 of DHBV PreS region of L surface antigen (72). As this region includes sequences not involved in CPD binding (aa 22-43) it is unlikely that CPD is the sole determinant of DHBV entry. Interestingly, mutations in PreS that ablate viral infectivity without affecting the PreS-CPD interaction map to aa 20-40 in PreS (73). As this region corresponds to the region of PreS responsible for species-specific cellular binding, it may represent a region responsible for binding to a co-receptor (72). As well, HHBV PreS efficiently binds duck CPD, indicating that lack of CPD binding is not responsible for HHBV's inability to infect pekin ducks. Fourth, expression of CPD in cells that are capable of supporting DHBV replication but refractory to DHBV infection does not confer susceptibility to a productive DHBV infection (15).

One such potential co-receptor for DHBV is DGD, the p protein of the duck glycine decarboxylase complex, a 120 kDa protein that was purified from duck livers using truncated L surface antigen- GST fusion proteins immobilised on Sepharose beads (92, 93). There are several lines of evidence that suggest that duck glycine decarboxylase (DGD) may function as a DHBV receptor/co-receptor. First, unlike CPD, the tissue distribution of DGD closely parallels that of the main sites of DHBV replication (93). High levels of DGD are only expressed in the liver, kidneys and pancreas, with low levels present in a variety of other tissues (92, 93). Second, the amino acids of the PreS region which interact with DGD, aa 98-102 of PreS, co-localise to a region containing several neutralising epitopes (23, 93, 181). Third, the three residues shown to comprise the DGD binding motif, ^{100}FRR^{102}, are highly conserved among different DHBV isolates (23, 93, 181). Fourth, DHBV with a disrupted DGD binding motif exhibits reduced infectivity *in vitro* (93). Fifth, PreS peptides covering DGD's binding site were found to interfere with DHBV infection *in vitro* (93). However, these peptides also contained the binding domain of CPD so it is unclear how much of the inhibition is due to the blocking of virus binding to DGD. Although DGD is thought to be primarily a mitochondrial-associated protein, labeling of surface proteins, immunofluorescence staining of PDHs and transient expression studies all show that DGD is expressed both in the cytoplasm and on the surface of cells (92).

One puzzling observation is that DGD only binds with high affinity to N-and C-terminal truncated forms of L surface antigen (93). Although binding to full-length L surface antigen has been demonstrated, it is much less efficient. Optimal binding has been mapped to peptides containing the PreS region aa 98-102, which coincides with the binding site for a neutralising monoclonal antibody (93). This suggests that the DGD binding motif is normally hidden by the surrounding PreS sequences but is made accessible by truncation at either the N or C-terminus. It was also observed that efficient

DGD binding required precise truncation at the PreS C-terminus. Constructs that terminated at PreS residue 102 bound DGD with high affinity while peptides that terminated at residue 104 did not (92, 93). It is possible that a proteolytic event and/or conformational change of PreS is required to expose the DGD binding site. The sequence surrounding residue 102 in PreS does contain a putative cleavage site for furin, which is responsible for the cleavage/activation of a number of viral envelope proteins. It is possible that while CPD functions as the initial binding receptor on the cell surface, DGD's role is primarily post-internalisation. CPD binding and internalisation could be followed by a proteolytic/and or conformational change in PreS which then facilitates DGD binding. DGD binding may be the trigger for viral-endosome membrane fusion which facilitates release of the DHBV capsid into the cytoplasm. This would explain why DGD-reactive PreS peptides do not inhibit DHBV infection as well as CPD-reactive PreS peptides (93). If DGD binding occurs post-internalisation then peptides would not have an inhibitory effect unless they were able to cross the plasma membrane. As well, the infectivity of DHBV variants in which PreS residues 103 or 104 are altered is substantially reduced (93). The variant's ability to replicate and secrete virions from transfected cells is not reduced when compared to wild-type DHBV and so it is unlikly that the reduced infectivity is due to defective replication. It is possible that altering residues 103 or 104 effects the ability of furin (or another endopeptidase) to cleave PreS, thereby preventing the activation of the DGD binding site. This would then prevent the completion of DHBV entry into the hepatocyte.

The exact role of DGD in DHBV entry, if any, remains to be determined. To date there have been no reports of expression studies to determine if DGD expression enables a non-permissive cell line to support a productive DHBV infection. The results of DGD and CPD co-expression studies will be of particular interest. Expression of CPD alone into the chicken hepatoma cell line, LMH, allows binding and internalisation of DHBV but does not result in a productive infection. As LMH cells are capable of supporting DHBV replication after transfection of the complete DHBV genome, the block likely involves a step related to DHBV entry. It is interesting to note that the duck and chicken glycine decarboxylases (CGD) have approximately 91% identical residues, with the exception of the N-terminus where the DGD contains additional amino acids (92). This region contains the putative mitochondrial targeting domain. CGD is localised to the mitrochondria whereas DGD is seen in both the cytoplasm and cell surface. The divergent N-terminal sequences may be responsible for the the different localisation patterns of the two proteins. This might explain why CGD expression in LMH cells does not result in a productive DHBV infection. The primarily mitochondrial localisation of CGD may prevent it from functioning as a co-receptor despite its high sequence homology with DGD.

There is also evidence that a third protein may be involved in DHBV entry. Guo *et al* described a 55 kDa protein isolated from duck liver cells using two monoclonal antibodies generated by immunising mice with duck hepatocytes (56). To date the identity of this protein is unknown. The mAbs partially blocked DHBV binding and also inhibited DHBV infection of PDHs. Neither Mab reacted with PreS, indicating that inhibition of infection was likely due to a specific interaction between the antibodies and a host cell surface molecule. The role of this protein in DHBV infection, like DGD, remains to be determined. The presence of the 55 kDa protein on the cell surface could not be directly demonstrated by immunofluorescence, indicating that the levels are possibly too low for detection. As well, it appeared to be expressed in most duck tissues. Neither of these observations necessarily rule out the 55 kDa protein as a potential DHBV receptor since cell surface levels of CPD and DGD are also relatively low and CPD expression is found in a variety of tissues.

1.3.2 Viral entry into hepatocytes.

Determining the exact mechanism of hepadnaviral entry into cells is proving to be as challenging as the identification of cellular receptors. The results seem to be consistent with two known models of virus entry. Entry of all enveloped viruses involves the fusion of the viral and cellular membranes, resulting in the release of the viral capsid and nucleic acid into the cytoplasm. Fusion is initiated by a viral envelope protein and can occur either at the cell surface, a mechanism employed by human immunodeficiency virus (HIV) and paramyxoviruses, or from within endosomes following receptor-mediated endocytosis, such as influenza virus entry. Viral envelope proteins contain fusion peptides that are normally buried within the protein. A conformational change is required to expose the fusion peptide, which can then insert into the cellular membrane and induce fusion. In the case of viruses such as paramyxovirus and HIV, binding of the virus to a specific cellular receptor or co-receptor induces the conformational change. This process ocurs at the cell surface and is independent of pH. With viruses such as influenza and Semliki Forest virus, the conformational change is induced by the acidic environment of the endosome following receptor-mediated endocytosis.

Lysomotropic agents, which raise the pH inside the endosomes, have been used to differentiate between these two mechanisms of viral entry. Fusion from within endosomes is sensitive to lysomotropic agents, because these agents prevent the acid-induced conformational change in the viral protein required for fusion. Conversely, fusion at the surface of the cell occurs at neutral pH and so is not affected by these agents. Several groups have used lysomotropic agents to differentiate between the two potential mechanisms to study DHBV entry, with conflicting results. Offensperger *et al* demonstrated that ammonium chloride and chloroquine effectively inhibited (119) DHBV infection of PDHs. The effect was greatest when the drugs were added before or at the time that virus was added to the cell cultures. These results suggest that DHBV entry occurs by receptor-mediated endocytosis, followed by acid-induced fusion of the viral and endosome membranes. However, results from a similar study showed that DHBV infection of PDHs was not affected by either ammonium chloride (79, 134) or monesin, another lysomotropic agent (134). In fact, in some cases DHBV replication was actually increased up to two-fold by the addition of these drugs (134). The reason for the conflicting results regarding DHBV sensitivity to lysomotropic agents is not clear. The concentration of ammonium chloride used in the studies was similar (20 to 30mM) to that sufficient to block entry of Semliki Forest Virus, which is known to enter cells in a pH-dependent manner (134). Rigg *et al* suggest that the extended exposure of cells to the lysomotropic agents used in the first study, up to two weeks, was inhibiting DHBV replication at a step other than entry, such as viral assembly. However, Offensberger *et al* demonstrated that the drugs have no effect on the on-going replication of DHBV present in the cells of congenitally infected PDHs (119). Rigg *et al* provided additional evidence that DHBV entry is pH-independent. For example, they found that DHBV infectivity was not inhibited by pre-treatment of the virions with low pH prior to exposure to hepatocytes (134). As well, attempts to induce fusion of DHBV bound to cells with low pH failed. DHBV remained bound to the cells but did not enter until it was exposed to neutral pH.

The finding that DHBV entry was likely pH-independent therefore suggested that entry was occurring via fusion at the cell surface. However, evidence shows that DHBV enters cells by endocytosis. Endocytosis is an energy-dependent process and inhibitors of ATP synthesis inhibit the uptake of viruses that enter by endocytosis. DHBV uptake was found to be inhibited in the presence of sodium azide and 2-deoxy-D-glucose, which inhibit oxidative phosphorylation and glycolysis, respectively (79). Further evidence that DHBV enters cells by endocytosis comes from studying the effects of various mutations of the DHBV receptor, CPD, on DHBV infection. PDHs were transduced with recombinant adenoviruses encoding various CPD mutants prior to the addition of DHBV to the cell cultures. It was found that arresting CPD at the cell surface, by deleting the cytoplasmic tail, blocked subsequent DHBV infection (13). This suggests that fusion of viral and cell membranes is not occurring at the cell surface but rather that endocytosis of the DHBV-CPD complex is required for DHBV entry. A stretch of acidic amino acids in the C-terminus of the cytoplasmic tail of CPD contains a putative casein kinase II recognition site which has been implicated in trafficking. It is thought that DHBV takes advantage of this by remaining bound to CPD post-endocytosis, allowing it to escape the lysosome. Deletion of the C-terminal tail results in accelerated transport of CPD to the lysosomal compartment rather than to its usual target of the Golgi network. Expression of this mutant CPD inhibited DHBV infection, presumably due to virus degradation in the lysosome. CPD has a relatively long half-life (days) and has the potential to cycle several times between the Golgi and cell surface without being subjected to lysosomal degradation.

Internalisation of DHBV into PDHs was found to be slow, taking up to eight hours at 37°C (134). There are two possible explanations as to why DHBV entry is slow. The first is the slow cycling rate of CPD. In antibody-uptake experiments, CPD cycling was shown to be relatively slow in cultured primary hepatocytes (13). This would explain why DHBV bound to cell surfaces remains susceptible to removal by acidic glycine buffer for up to 8 hours at 37°C (134). The second explanation is that a proposed conformational change of L surface antigen, which may be required for complete DHBV entry, is relatively slow (discussed in section 1.4.4).

In summary, the current proposed model for DHBV entry involves receptor-mediated endocytosis (13). Following this, DHBV and CPD are thought to be transported to an endosome where interaction with a second receptor possibly occurs, resulting in fusion between the viral and endosome membranes and release of the DHBV nucleocapsid into the cytoplasm.

1.4.3 Nucleocapsid disassembly and nuclear import of viral DNA.

Once the DHBV nucleocapsid is released into the cytoplasm, the viral DNA must make its way into the nucleus. The mechanism underlying the nuclear import of viral DNA is poorly understood for hepadnaviruses in general. NLSs have been identified in both HBV and DHBV core proteins. The NLS in the DHBV core protein is located in the C-terminus, a region previously shown using sequence specific antibodies to be exposed on the surface of nucleocapsids (105). It is a classic NLS, consisting of a single monopartite stretch of basic amino acids, encompassing the following DHBV core sequence: 214-PRRRRKVK-220. Amino acid substitution experiments indicate that this sequence is essential for the targeting of core protein to the nuclear pore. These results indicate that this sequence functions to direct the nucleocapsid to the nuclear membrane for import of the viral genome. The mechanism of delivery of the viral DNA into the nucleus is not yet understood. Protein-free DNA is only poorly imported into the nucleus, indicating that one or more viral proteins are involved (77). Studies of WHV suggest a mechanism where nucleocapsids are directed to the nuclear membrane where they bind in an ATP-independent manner (77). The polymerase protein is sufficient for mediating the transport of the viral DNA across the nuclear membrane. The import of the viral polymerase-DNA complex into the nucleus is an ATP-dependent process and likely occurs after disassembly of the nuclear-membrane bound capsids (77). It is not clear whether capsids are partially disassembled in the cytoplasm prior to nuclear membrane binding or if disassembly occurs after binding but prior to or during nuclear import. A serine or threonine-proline kinase recognition motif has been identified in core protein at a location that is required for the assembly of core dimers into capsids (5). There is some evidence to suggest that phosphorylation at the threonine 174 position within this motif could trigger nucleocapsid disintegration, as indicated by a reduced accumulation of core protein (5). It is possible that phosphorylation induces a conformational change which leads to a de-stabilisation of a critical core dimer-dimer interaction.

1.3.4 Formation of covalently closed circular DNA (cccDNA).

Once the viral DNA has entered the nucleus, several modifications occur to form cccDNA, the template for all further viral replication. The terminal protein and the RNA primer attached to the 5' ends of the (-) and (+) strands, respectively, are removed (1). As well, the (-) strand is trimmed to remove the redundancy, the (+) strand completed and the ends of both strands ligated to form cccDNA (1). The mechanism of this process is largely unknown. Inhibiting the viral polymerase activity with a nucleoside analogue has no influence on the genome modification (80), indicating that this enzymatic activity is not required.

The initial cccDNA is generated by the conversion of the relaxed circular (rc) viral DNA present in nucleocapsids from virions that have recently entered the cell. This initial population is then amplified to a steady-state pool of 20-30 cccDNA/cell by an intracellular pathway involving directing newly formed nucleocapsids back to the nucleus (2, 164). The RC genome present in these capsids is converted to cccDNA. This process is at least partially regulated by the levels of the L surface antigen (91, 151, 152). Early in infection, when cccDNA levels are low, the amount of L surface antigen expressed is also minimal. However, as the levels of cccDNA increase so do the levels of L surface antigen present. Once a particular level of L surface antigen is reached, its interaction with mature nucleocapsids directs these to the secretory pathway. This inhibits the nucleocapsids from targeting the nucleus and hence halts further amplification of the cccDNA pool.

The cccDNA is stably associated with proteins to form a viral minichromosome in the nucleus of infected cells (118), similar to what has been shown for other viral genomes such as SV40 (55), adenovirus (156), HSV-1 (only during latency) (36) and cauliflower mosaic virus (120). The minichromsomes of DHBV cccDNA are composed of nucleosomes that contain elements of approximately 146bp of protein-bound DNA and 5 bp of linker DNA which results in a 150bp ladder following micrococcal nuclease digestion (118). The cccDNA molecules exist as a heterogenous population in terms of superhelicity and nucleosome number and it is possible that the variation in number of nucleosomes and linker DNA lengths play a role in the regulation of transcription. For example, cccDNA molecules with fewer nucleosomes and more extensive lengths of linker DNA may be more transcriptionally active than cccDNA molecules that are completely organised into nucleosomes.

Because cccDNA serves as the template for DHBV replication, the stability of the cccDNA pool is of considerable interest with regard to antiviral therapy. The cccDNA pool is relatively resistant to the effects of nucleoside analogues (111) and so determining the half-life is crucial for monitoring the success of antiviral therapy. An *in vitro* analysis of the half-life of cccDNA present in infected PDHs was done by measuring the decay of unlabelled cccDNA following a pulse-chase experiment using 5-bromo-2-deoxyuridine to label the cccDNA. The unlabelled cccDNA decayed with a half-life of 3-5 days (30). However, this result is not consistent with the observation that withdrawal of lamivudine after a continuous, year long treatment results in an rapid re-bound of viremia in DHBV-infected ducks (Tyrrell, personal observation). Similar results are obtained when chronic HBV carriers are treated with lamivudine (25, 38, 58). A much different half-life was obtained in a study using DHBV-infected ducks in which viral replication, and hence new cccDNA synthesis, was effectively inhibited by treatment with lamivudine and a dideoxyguanosine prodrug (2). The decay of the cccDNA pool in the nucleus of DHBV-infected ducks was measured using serial liver biopsies and a quantitative PCR assay specific for cccDNA (3). In contrast to the *in vitro* study, the half-life of cccDNA was found to be quite long, between 35-57 days. A similar study done in the WHV model using the nucleoside analogue L-FMAU (L-2'-deoxy-2'flouro-5-methyl-1-β-D-arabinosyluracil) to suppress viral replication revealed a similar half-life of between 33 and 50 days (184).

1.3.5 Viral transcription.

The pool of cccDNA serves as the template for viral transcription by the host RNA polymerase II. Three major polyadenylated mRNA species are produced in DHBV replication (16). All the following nucleotide numbers are based on the Mandart numbering system of the DHBV genome (109). These include a 3.5 kb transcript which contains the complete viral genome plus an additional 270 nt redundancy including nucleotides 2530/2531 up to 2800 (16). This transcript, termed the pregenomic RNA, serves as the mRNA for the translation of both the core and polymerase proteins as well as the template for the synthesis of viral DNA by reverse transcription. The remaining 2.3 kb and 2.1 kb transcripts serve as mRNAs for the translation of the L and S envelope proteins, respectively (16). Figure 1.4 shows the relationship between the viral ORFs and RNA transcripts.

RNA molecules are transcribed from the (-) strand of viral DNA, are unspliced and share a common 3' terminus (16). The 5' ends of the transcripts for the pregenomic/core, L and S surface antigen genes map to positions 2530/2531, 732/740 and 985, respectively. The promoters for both the pregenomic RNA and L surface genes contain the classic TATA-box while the promoter for the S gene lacks a TATA-box. However, in the position where the TATA-box is normally present, -25 relative to the transcription start site, there is a consensus binding site for the transcription factor hepatocyte nuclear factor 3 (HNF3). This sequence is essential for S surface antigen gene transcription and may contribute to the S promoter's dependence on differentiated hepatocyes (32, 98). The transcription start site for an additional minor RNA species, corresponding to the PreC mRNA which encodes the so called "e" antigen, initiates at nucleotide 2448 (141). It was found that sequences between the PreC mRNA and C mRNA initiation sites contain a regulatory element that is capable of downregulating PreC transcription. Although its biological significance is unclear, the motif is conserved among avian hepadnaviruses and its presence is consistent with the low levels of PreC mRNA found in infected livers, approximately 1-5% of C-mRNA/pregenome (141). The same motif is present in a second location a few nucleotides downstream of the 5'end of the C mRNA but for reasons that are unclear it does not prevent efficient transcription from the C promoter.

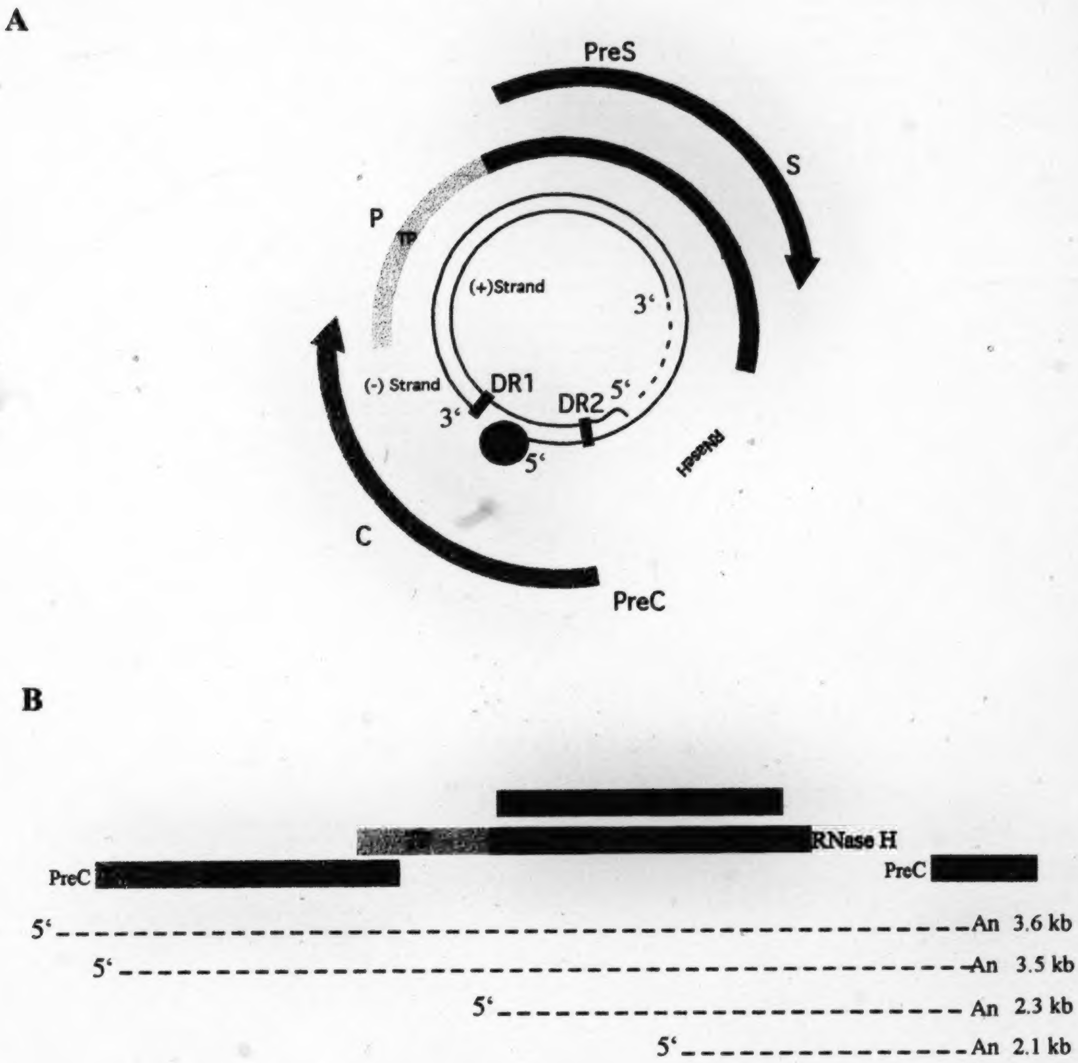

A
PreS
S
P
TP
(+)Strand
3'
(-) Strand
DR1
3'
DR2
5'
5'
RNaseH
C
PreC
B
RNase H
PreC
PreC
5'
An 3.6 kb
5'
An 3.5 kb
5'
An 2.3 kb
5'
An 2.1 kb

Figure 1.4: Relationship between DHBV genome, ORFs, and mRNA transcripts. **A.** Relationship between the DHBV genome and the ORFs of the precore, core, polymerase and envelope proteins. **B.** Relationship between the ORFs and the mRNA transcripts.

The 3'end of all transcripts map to nucleotide 2800, which is located near the signal sequence AAUAAA (2772-2777) known to be involved in 3' mRNA processing and polyadenylation (16). It is interesting that the polyadenylation site is located within the core ORF and is approximately 240 nucleotides downstream of the transcription start site for both the PreC mRNA and the pregenomic RNA transcripts. This suggests that the polyadenylation signal is ignored on the first pass to produce the full-length PreC and pregenomic transcripts. One possible reason for this is the presence of sequences upstream of the pregenomic RNA initiation site that influence secondary structure of the nascent RNA and thus recognition of the polyadenylation signal. Another potential mechanism involves a sequence identified as *pet*, or positive effector of transcription (67). *Pet* is a cis-acting element in the 5' end of the pregenomic RNA (nucleotides 2562-2616) that is required to suppress premature termination of pregenomic RNA transcription in a region 500-1200 nucleotides downstream of the transcription initiation site. The *pet* sequence may be the hepadnavirus equivalent of the HIV TAR sequence. TAR is located in the 5' end of the HIV RNA, just after the transcription initiation site, and functions through the formation of a specific RNA secondary structure which is bound by the HIV tat protein and several cellular cofactors (114). This increases the processivity of the RNA Polymerase II complex, resulting in significantly higher steady-state levels of viral mRNAs. Similar to TAR, inversion of *pet* destroys its activity, suggesting that it functions through a specific RNA secondary structure (67). While there is no evidence that a viral protein binds *pet*, it is possible that cellular proteins bind to *pet* and either stabilize the nascent RNA or allow the RNA polymerase to read through the polyadenylation signal.

To date, several groups have independently identified one enhancer region in the DHBV genome. It maps to a region just upstream of the pregenomic RNA CAP site at nucleotide 2531 and within the the 3' end of the polymerase ORF at nucleotides 2159-2351, (32), 2172-2355, (98) and 2155-2306 (141). The enhancer activates transcription from both the pregenomic/core and surface promoters but has no effect on transcription from the PreS promoter (98). The activity of the enhancer is significantly higher in cells of hepatic origin, although it functions in non-liver cells as well (98). In addition, enhancer activity is greatest in highly differentiated liver cells (141). It is likely, therefore, that the nature of the enhancer elements accounts at least partially for the tissue specificity of DHBV replication. The DHBV enhancer is not species specific, however, so the species specificity of DHBV infection is likely due to other factors, such as virus entry.

The enhancer region has been at least partially characterised and includes binding sites for both liver-specific and ubiquitious transactivating factors. The liver specificity of the enhancer is due to binding sites for one C/EBP, one hepatocyte nuclear factor 1(HNF1), and three HNF3-binding sites (95). The enhancer also includes F3, a sequence that is similar to that recognized by the uniquitous factor EF-C, which is essential for transcriptional activation (95). C/EBP, which is involved in the transcriptional control of albumin, can also function as a repressor (95). HNF1 is essential for the activation of many liver-specific genes and also appears important for the DHBV enhancer activity, at least *in vitro* (95, 99). HNF1 binding to the enhancer can inhibit binding of HNF3 to a less than optimal consensus sequence at an adjacent site (31). Interestingly, individual mutations within the enhancer HNF1 and HNF3 binding sites which decreased viral RNA synthesis *in vitro* did not have a major effect on viral replication either *in vitro* or *in vivo* (99). However, multiple mutations within the enhancer had a greater effect. This is consistent with a synergistic effect of transcription factor interaction with the enhancer region.

Hepadnaviral RNA transcripts are not normally spliced (16). Since RNA splicing and cytoplasmic transport are tightly- linked processes in eukaryotic cells, there must be separate mechanisms to ensure efficient nuclear export of viral transcripts. The Rev-RRE (rev-response-element) system in HIV functions to regulate the nuclear export of unspliced and partially spliced HIV transcripts and allows expression of proteins expressed from these transcripts (34, 108, 129). A similar motif is present in the HBV genome, termed post-transcriptional regulatory element (PRE). It was originally identified in the S transcript and was found to be necessary for its export from the nucleus to the cytoplasm to allow expression of the surface antigen protein (68). The PRE is a region approximately 570 nucleotides long and is present, at least partially, in the 3' terminus of all viral transcripts (69). The PRE is composed of three separate regions, including two highly conserved stem loops which function synergistically and are necessary for PRE activity (39, 145). The PRE has been shown to specifically bind at least two cellular proteins (70). One of these cellular proteins has been identified as glyceraldehyde-3-phosphate dehydrogenase (GADPH) whose role in PRE-mediated export is not yet understood but it may be involved in the nuclear export of tRNA (182). The other protein is polypyrimidine tract binding protein (PTB), an RNA-binding protein which shuttles between the nucleus and cytoplasm and which is thought to play a role in mRNA export (183). Mutants of PRE with decreased PTB-binding show a decreased ability to export intronless mRNAs. In addition, both the export of PTB from the nucleus and PRE function are blocked by a mutant form of Ran binding protein 1 (RBP-1) but not leptomycin B, an antibiotic that inhibits the function of proteins that export proteins with NLSs. PTB likely facilitates the nuclear export of unspliced HBV transcripts by simultaneously interacting with the PRE and cellular proteins involved in nuclear export pathways. Although a PRE has yet to be identified in DHBV, one has been identified in WHV, indicating that this element is conserved at least within the mammalian hepadnaviruses (40).

It is interesting to note that all transcription signals, including promoters, enhancer, *pet* sequence, PRE and the polyadenylation signal are located within viral ORFs.

1.3.6 Translation of viral gene products.

The core ORF encodes two separate proteins, e antigen and core protein which are expressed from separate mRNA transcripts. The secreted form of precore protein, e antigen, is translated from a minor mRNA transcript that originates upstream of the C mRNA/ pregenomic RNA (140). Translation of precore protein is initiated at the first of two potential start AUG codon, at nucleotide 2518. The PreC region of the transcript contains a signal sequence which targets the precore protein to the ER, where the protein is modified by N-linked glycosylation at one or two sites (137, 139, 140). The precursor precore protein is further modified by both N-terminal and C-terminal proteolytic processing (137, 139). The C-terminal cleavage, which removes the strongly basic region, occurs post-glycosylation and possibly during passage through the Golgi apparatus as the protein is exported from the cell (137). The C-terminus of the precore protein appears to be required for intracellular transport as precore mutants lacking this domain are glycosylated but not secreted (137). DHBV e antigen differs from core protein in that it contains additional sequences at the N-terminus and is truncated at the C-terminus. It is present in the serum of infected animals as 33, 30 and 27 kDa proteins which represent the double glycoslylated, single glycosylated and unglycosylated forms of the protein, respectively. In addition to the e antigen, there appears to be a membrane form of precore protein with unusual topology in that the C-terminus is exposed on the surface of the cell. It is speculated that this membrane expression is due to proteins which are not C-terminally processed, which is required for secretion.

Core protein is expressed from the pregenomic RNA transcript which functions both as a template for genomic DNA synthesis and as an mRNA for the expression of the core and polymerase proteins. Translation of core is initiated from the second AUG of the core ORF, at nucleotide 2648, and gives rise to an approximately 31 kD protein (16). The core protein is modified by phosphorylation at a minimum of three serine residues located at amino acid positions 245, 257 and 259, a threonine at 239, and a potential phosphorylation at threonine 174 (5, 180). The state of phosphorylation/de-phosphorylation at these positions appears to regulate the multiple functions of the core protein (179). A region in the pregenomic RNA which corresponds to the 3' end of the core ORF, spanning nucleotides 401 to 870, interacts with the DHBV polymerase protein, causing selective inhibition of core mRNA translation *in vitro* (64). The encapsidation signal of the pregenomic RNA is present in the core ORF and it is speculated that this polymerase-RNA interaction may displace translating ribosomes on the C mRNA, thus exposing the encapsidation signal and facilitating nucleocapsid assembly.

The largest ORF in the DHBV genome encodes the polymerase protein. The 5' end of the polymerase ORF overlaps 305 nucleotides at the 3' end of the core ORF (20). Because no mRNA transcript corresponding to the polymerase gene alone has been identified, it is assumed that both proteins are expressed from a single transcript, the pregenomic RNA, but in different reading frames with the polymerase translation in a +1 frame relative to core (20). The gag and pol genes of retroviruses are similarly organised and pol is expressed as a nucleocapsid-polymerase fusion protein as a result of ribosomal frameshift during translation of the nucleocapsid protein (74). However, this mechanism does not appear to be utilised for the expression of DHBV polymerase. First, no core-polymerase fusion protein has been detected (18, 20, 138). Second, polymerase expression is not affected by frameshift mutations in the core gene which cause premature termination of translation of the core protein upstream from the core-pol overlap region (18, 20).

Translation of polymerase is initiated at the first AUG in the polymerase ORF, at a position that is 660 nt downstream of the 5' terminus of the pregenomic transcript (20). Evidence for this comes from two experiments: 1) mutation of this AUG to ACG eliminates expression of polymerase, 2) introduction of stop codons 3' but not 5' to this position eliminates polymerase expression (20).

It is not clear how ribosomes gain access to this internal AUG. To date, there is no evidence of sequences (45) that are capable of mediating internal entry of ribosomes similar to the internal ribosome entry site (IRES) present in poliovirus mRNA transcripts (123, 124). As well, hepadnavirus polymerase translation is likely CAP-dependent as polymerase expression is decreased 90% in the presence of poliovirus, which inhibits CAP-dependent translation initiation (18). One possible mechanism of this internal translation initiation is ribosome scanning, where a few ribosomes bypass all potential AUGs upstream of the polymerase AUG. However, conventional ribosome scanning seems unlikely for two reasons. First, despite the presence of 14 additional AUGs in all three reading frames upstream of the polymerase AUG, *in vitro* translation initiation of polymerase appears to be almost as efficient as that of the core protein which is initiated at the 5' terminus of the RNA transcript (18). This is in contrast to translation initiation of most eukaryotic transcripts, where only the AUG codons closest to the 5' end are efficiently utilized for initiation (81). Second, translation of polymerase is for the most part independent of core translation (18, 20). This is also inconsistent with a model of ribosome scanning because translation initiation of downstream AUGs should be either decreased or increased by mutations that enhance or weaken the upstream initiator, respectively. Studies of expression of HBV polymerase have led to a unique ribosome scanning model in which the polymerase gene is translated from ribosomes scanning from the capped 5' end of the pregenomic RNA (45). The AUGs upstream of the polymerase AUG are skipped by the ribosome because they are in a suboptimal initiation context. In addition, the translation of a small ORF overlapping the C gene allows the by-pass of a strong out-of-frame AUG that would prevent further down-stream ribosome scanning. After translation of this small ORF, the ribosomes then re-initiate translation at the polymerase AUG. However, there are substantially more AUG codons present in the pregenomic transcript upstream of the duck polymerase AUG than in the human transcript, 14 as compared with 4 (45). It remains to be determined, therefore, if this same mechanism is applicable to expression of polymerase proteins of all the members of the hepadnaviridae family.

The two envelope proteins of DHBV, L and S surface antigen, are translated from separate mRNA transcripts as already discussed (16). Translation of the L surface antigen is initiated from the first AUG present on the PreS/S mRNA transcript, at nucleotide 801, resulting in a 36 kDa protein. Translation of the S surface protein is also initiated at the first AUG of the S mRNA transcript, at nucleotide 1287, resulting in a 17 kDa protein. Unlike the HBV envelope proteins, DHBV large and small envelope proteins are not glycosylated. The L envelope protein is modified by myristylation at an N-terminal glycine residue and this modification is essential for virus infectivity (107). In addition, the L surface antigen is phosphorylated at a serine residue in the PreS domain (51).

The L surface antigen mRNA transcript of DHBV contains four additional conserved AUGs whose functions remain unknown. There is evidence that translation of minor L surface antigens are initiated at these internal AUGs and that these proteins are componants of the viral particles (44). L surface antigen proteins of 35, 33, and 30 kDa have been identified and correspond to translation initiation at internal AUG codons located at nucleotides 825, 882 and 957, respectively. However, in contrast to the major 36 and 17 kDa envelope proteins, these proteins are not required for viral infectivity and have no effect on virus production in ducklings. The fact that the internal AUGs are conserved in both DHBV and HHBV suggest some role of the minor envelope proteins in infection, perhaps in establishing and maintaining a chronic infection. Another explanation is simply that methionines are required at these positions and that the AUG codons do not function as translation initiators.

The genomes of mammalian hepadnaviruses contain a fourth ORF, called X gene, which encodes a protein of 17 kDa (1). This ORF overlaps with the 3' terminus of the polymerase and 5' terminus of the core ORFs. Along with the mammalian hepadnaviruses, most avihepadnaviruses appear to have a conserved X gene. The genomes of hepadnaviruses isolated from snow geese (21), grey heron, (117), Ross geese (117) and white storks (22) show an ORF in a position analogous to that of the X gene in mammalian hepadnavirus

Although the exact function of the X protein in relation to hepadnavirus replication/infection is not known, it appears to be essential for the establishment of infection *in vivo*, at least in the case of WHV (185). The X protein is expressed *in vivo*, as shown by the presence of anti-X antibodies in HBV-infected individuals and immunostaining of infected liver tissue (61). It is localised primarily to the cytoplasm but is found in small amounts in the nucleus (144, 149). The X protein is a moderate transactivator of a variety of both cellular and viral promotors, acting mainly through protein-protein interactions with several componants of the basal transcription machinary (96, 132). It also stimulates several related cytoplasmic signal transduction pathways, including the Ras-Raf-MAP kinase, the stress-activated protein kinase (SAPK)-NH2-terminal-Jun kinase (JNK), and JAK/STAT-signalling pathways (11, 33, 89). X protein stimulates Ras activity, possibly by activation of Src kinases which are upstream activators of Ras GTPases. Src kinases are also activators of the JAK-STAT signaling pathway and activation of these kinases may affect numerous signalling pathways. Chronic DHBV infection in ducks is not associated with the development of hepatocellular carcinoma, and this, in addition to X protein's effect on transcription and signalling pathways, has led to the speculation that the X protein has oncogenic potential.

In contrast to the mammalian and other avihepadnaviruses, DHBV was generally thought to lack an X gene. There is some recent evidence, however, that suggests that there may be an X gene present in the DHBV genome. The size and position of the core gene in DHBV and HHBV is comparable to the size of the combined core and X genes of HBV (43). In addition, there is weak sequence homology between the middle region of DHBV core and the X gene in HBV (43). However, there is no report of X-like functions associated with DHBV core protein. Sequence analysis of numerous DHBV isolates revealed the presence of a potential ORF in the same position as the X ORF present in all other hepadnaviruses (22). This putative ORF initiates at nucleotide 2289 and ends at nucleotide 2633. However, unlike the other hepadnaviruses, the putative DHBV X ORF would use one of several non-conventional translation initiation codons. The region corresponding to this ORF is highly conserved among different DHBV isolates and also has considerable homology with the X-like sequences of SGHBV(90%), HHBV and RGHV (40-50%). It remains to be determined how or if a DHBV X protein is produced. A promoter has been identified upstream of the ORF and one possibility is that X protein is expressed from a terminally redundant RNA transcript similar in size to the pregenomic RNA. This transcript would initiate upstream of the DHBV X ORF and terminate only after the second pass of the single RNA processing site present in the DHBV genome. Translation of X protein could then be initiated from one of several potential non-AUG start sites. Another alternative is that X protein is translated from a minor RNA transcript that has not been detected due to insensitivity of current detection systems.

There is direct evidence for the existance of a DHBV X protein expressed from this potential ORF. Antibodies generated against either peptides corresponding to the putative DHBV X protein or bacterially-expressed DHBV X-protein detected a 12kDa protein expressed at low levels in DHBV-infected but not uninfected liver extracts (22). The DHBV X protein, when expressed *in vitro*, was found to have several similarities to HBV X protein. First, expression of the DHBV X-like ORF had no effect on viral protein or DNA synthesis, or steady-state levels of viral RNA transcripts. Second, the DHBV X protein is localised primarily in the cytoplasm, with strong staining near the nuclear membrane. Third, the DHBV X protein stimulated promotors of both cellular and viral origin and this activation was dependent on the Ras-Raf-MAP kinase signalling pathway. As with the X proteins of all hepadnaviruses, the function of the X protein in viral replication/infection remains unclear.

1.3.7. Viral RNA encapsidation and assembly of pre-initiation complex

Virus assembly is initiated by the interaction of the polymerase protein with the *cis*-acting packaging signal, epsilon, present at the 5' end of the pregenomic RNA. It is a complex interaction involving multiple cellular proteins and conformational changes in both the viral polymerase protein and epsilon. Epsilon is a key element in the encapsidation/assembly process. It is involved in at least three critical steps of hepadnaviral replication: encapsidation of pregenomic RNA, maturation of the polymerase to an enzymatically active form, and the site of initiation of reverse transcription. The first two roles of epsilon are discussed in this section while the third role is discussed in section 1.3.10.

Due to the terminal redundancy of the pregenomic RNA, epsilon is present in both the 5' and 3'termini of the pregenomic RNA. However, only the 5' copy is used as a packaging signal since deletion of the 3' epsilon has no effect on viral replication (63). In DHBV, the 5' copy of epsilon is located approximately 35 nucleotides downstream of the cap site of the pregenomic RNA, starting at nucleotide 2562-2652 (17, 63). Like epsilons of other hepadnaviruses, this region is crucial for encapsidation of the pregenomic RNA (17, 63). Studies have shown that the overall secondary structure of epsilon is similar to that of other hepadnaviruses, with an upper and lower stem separated by a bulge and an apical loop (8). Investigation of the sequence and structure-specific elements that are crucial for the polymerase-epsilon interaction indicate that both the overall structure, particularly the bulge, and certain key residues are important (10). Some residues important for polymerase binding have been identified in the loop and are presumed to have direct contact with the protein as their substitution eliminates polymerase binding. Conserved residues adjacent to the loop also appear to be involved in the interaction. The polymerase prefers to bind to epsilon present on its own mRNA during the encapsidation process as evidenced by the observation that pregenomic transcripts expressing the polymerase are encapsidated more efficiently than transcripts lacking the polymerase (63). The region of the polymerase protein which interacts with epsilon has so far not been identified.

With at least four viral transcripts present in the cytoplasm, there must be a mechanism to ensure that the pregenomic transcript is selectively encapsidated. One is the requirement for epsilon at the 5' terminus of the transcript (63). This effectively eliminates the selection of the transcripts encoding the envelope proteins which do not contain the 5' copy of epsilon. However, although both the pregenomic and precore antigen transcripts contain epsilon at their 5' terminus, only the pregenomic transcript is encapsidated. Studies of GSHV suggest that active translation from the precore start AUG codon, which is upstream of epsilon, suppresses recognition of epsilon by the polymerase protein (115). Indeed, when the precore AUG is inactivated, these transcripts are encapsidated. The pregenomic transcript does not contain the precore AUG and so ribosome-mediated suppression does not occur. The precore transcript is also present in extremely low levels compared to the pregenomic transcript, approximately 1%, and so this likely also contributes to the selective encapsidation of the pregenomic RNA (141). In addition, studies of HBV have indicated that simultaneous recognition of the 5' CAP structure and epsilon by the polymerase protein is required for encapsidation (75). It is speculated that the polymerase interacts with one of the cap-binding factors. This explains why epsilon must be in close proximity to the CAP structure for successful encapsidation and likely accounts for the inability of the 3' epsilon to function as an encapsidation signal. In summary, there appears to be several mechanisms to ensure that the proper RNA transcript is encapsidated.

In addition to the epsilon sequence, a downstream RNA element is required for DHBV pregenomic RNA encapsidation, located at nucleotides 551 to 719 (17). This region does not contain any apparent structure or sequence homology with epsilon and its role in encapsidation remains unclear. The same domain (with regard to sequence) in HBV is not required for encapsidation, indicating that mammalian and avihepadnaviruses may employ different mechanisms of RNA packaging.

Cellular proteins are also involved in the assembly of the pre-initiation complex. The heat shock protein Hsp90, a cellular chaperone, has been shown to be required for the interaction of the polymerase with epsilon RNA (65, 66). It presumably stabilizes the polymerase in a conformation that is competent for epsilon binding. Also involved is p23, a phosphoprotein required for Hsp90 function. It interacts with Hsp90 and also appears to be able to bind the polymerase directly, independently of Hsp90. Hsp70 and its partner Hsp40 are also required for the interaction of the polymerase with epsilon but do not appear to be stable components of the polymerase complex. ATP hydrolysis, likely provided by Hsp70, also plays an essential role. Binding of the polymerase to epsilon does not facilitate the release of Hsp90 from the complex, which is unusual as the interaction of Hsp90 with its substrate is usually transient. There is some evidence that at least p23, and possibly Hsp90, are incorporated into the nucleocapsid by their association with polymerase.

Analysis of the polymerase binding/priming ability (ability of epsilon to support polymerase-primed reverse transcription) of mutant epsilon sequences indicates that the interaction likely involves a conformational change of the RNA. A difference in secondary structure has been demonstrated between free and polymerase-bound epsilon (9). Specific RNA-polymerase contacts is accompanied by a partial opening of the upper stem of epsilon. The secondary structure of a priming-incompetent epsilon variant is not significantly altered when bound to polymerase. This indicates that the structural rearrangement is essential for the formation of a replication-competent complex. The requirement for a conformational change in epsilon for priming activity may be related to the fact that upon binding to epsilon, the polymerase also appears to undergo a structural change. It was initially observed that expression of functional DHBV polymerase protein either in *Saccharomyces cerevisiae* or by *in vitro* translation required the interaction of the polymerase with epsilon during or shortly after translation (157). In the absence of epsilon, the polymerase was enzymatically inactive (157). It was subsequently shown that the reverse transcriptase domain of polymerase acquired resistance to proteolysis by a number of proteases when bound to epsilon, implying a conformational change upon epsilon binding (157, 159). The ability of the polymerase to adopt this epsilon-dependent protease resistant structure correlates with evidence for DNA priming and reverse transcription. It appears that the interaction of the polymerase with epsilon and the resulting conformational changes in both the RNA and reverse transcriptase domain are required for the activation of the polymerase enzymatic activity. This epsilon-dependent maturation of the polymerase could function to sequester the active reverse transcriptase and prevent it from targeting cellular messenger RNAs.

1.3.8 Assembly of nucleocapsids.

Following the polymerase's interaction with the pregenomic RNA via epsilon, the core protein interacts with polymerase to initiate the assembly of the nucleocapsid. The formation of nucleocapsids thus occurs in at least three steps (170). First, the polymerase binds to the epsilon structure at the 5' terminus of the pregenomic RNA. Second, this interaction then triggers core protein dimers to interact with the polymerase-epsilon pre-initiation complex. There is a slight, though distinct, bias towards a *cis*-preferential recruitment of the initial core proteins to the polymerase-pregenomic RNA complex (170). Finally, the core protein dimers multimerize to form the capsid shell. Subsequent reverse transcription and DNA synthesis occurs within the nucleocapsid particles in the cytoplasm and is discussed in the following section.

1.3.9 Overview of DNA replication.

Hepadnaviruses employ a unique mechanism of viral DNA synthesis which bears similarities to the replication of both retroviruses and cauliflower mosaic virus (1). DHBV DNA synthesis occurs within the cytoplasmic nucleocapsids and involves the following major steps (depicted in Figure 1.5):

i) Protein priming of reverse transcription by the polymerase protein.
ii) Reverse transcription of a 4-nucleotide DNA primer (5'-GTAA-3') corresponding to the bulge sequence (5'-UUAC-3') of the 5' copy of epsilon
iii) Transfer of this short DNA primer to a complementary sequence present in DR1 at the 3' end of the pregenomic RNA.
iv) Completion of (-) strand DNA synthesis and degradation of the pregenomic RNA template.

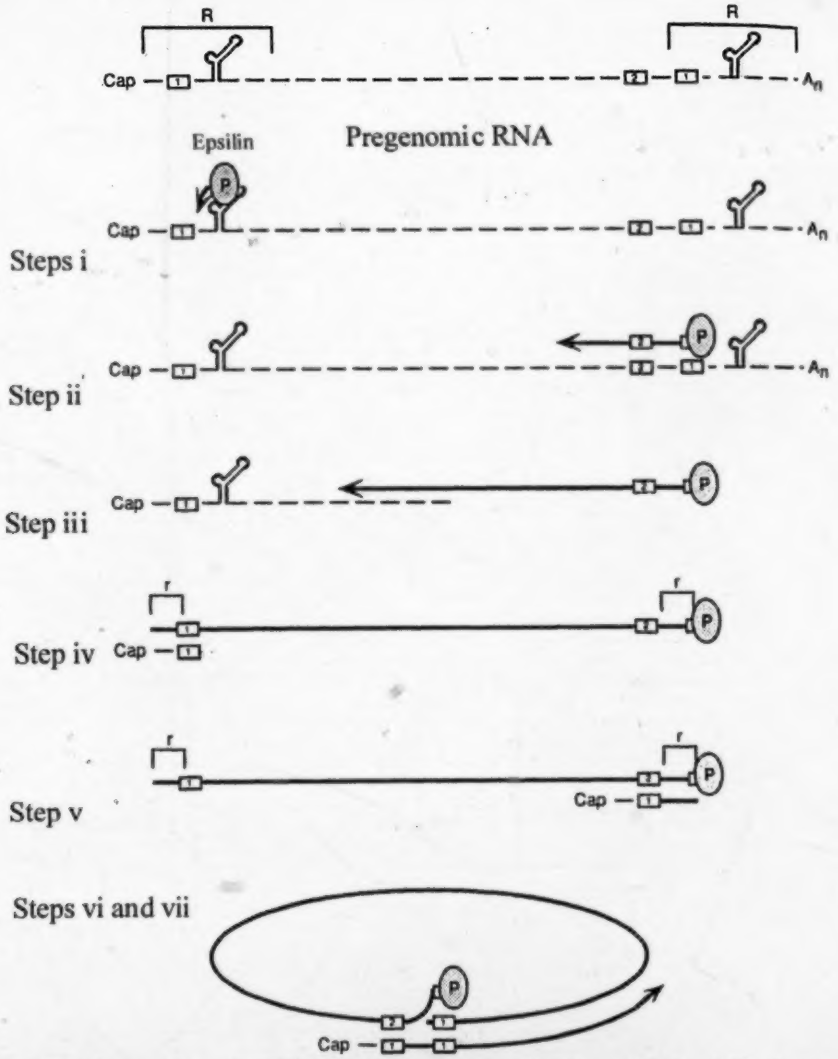

R
R
Cap
1
2
1
An
Epsilin
Pregenomic RNA
P
Steps i
Step ii
Step iii
Step iv
Step v
Steps vi and vii
r
r
P
Cap

Figure 1.5: Schematic of DHBV genome replication (Adapted from Fields Virology, 4th Edition). i) Polymerase protein binds to epsilon at the 5'end of the pregenomic RNA and synthesises a 4-nucleotide DNA primer. ii) Transfer of this DNA primer to a complementary sequence in DR1 at the 3'end of the pregenomic RNA. iii) Completion of the (-) strand DNA and degradation of the pregenomic RNA template. iv) Generation of a RNA oligomer by RNase H activity of the polymerase. v) Transfer of the RNA oligomer to DR2 present on (-)strand DNA and synthesis of primer for (+) strand DNA. vi) Transfer of the (+) strand DNA primer to DR1 present at the 3' end of the (-) strand DNA, resulting in the circularisation of the genome. vii) Synthesis of the (+) strand DNA using the (-) strand DNA as a template. P, polymerase, DR1and DR2, direct repeat 1 and 2, R, terminal redundancy, An, polyadenylation tail, dashed line, RNA, solid line, DNA

iv)Transfer of a short RNA oligomer generated by RNaseH activity to DR2 present on (-) DNA strand, where it anneals and initiates synthesis of the primer for (+) DNA strand.

v) Transfer of this (+) strand DNA primer to DR1 present at the 3' end of the (-) strand DNA, resulting in circularisation of the genome.

vii) Synthesis of (+) DNA strand using (-) DNA strand as template.

1.3.10 Priming of reverse transcription and (-) DNA strand synthesis.

The polymerase protein of hepadnaviruses is unusual in that a domain of the protein acts as the primer for DNA synthesis. This differs from other viruses such as adenoviruses in which the protein primer and polymerase are separate proteins (1). Early studies demonstrated that viral DNA present in nucleocapsid cores of infected cells was covalently attached to protein at the 5' terminus of the (–) strand DNA (12). Immunoprecipitation experiments showed that viral DNA could be immunoprecipitated with anti-polymerase antibodies, indicating that the protein attached to the viral DNA was the polymerase protein. Protease experiments later suggested that it was the the N-terminus of the polymerase, the terminal protein domain, that was linked to viral DNA (7). Wang *et al* was the first to provide evidence that the polymerase primed DNA synthesis by demonstrating that *in vitro* translated polymerase became labelled when incubated with radiolabelled dGTP (173). Protease studies and amino acid substitutions later determined that it was a tyrosine residue which provided the hydroxyl group for the creation of a phosphodiester bond with dGTP (175, 186). The tyrosine is present in the center of the terminal protein domain and it is speculated that it is positioned towards the active site of the polymerase to provide the hydroxyl group for priming of reverse transcription.

Following the covalent attachment of the first nucleotide to the polymerase, 3 additional nucleotides are then attached sequentially to dGMP to form the 5'end of the (-) strand DNA (172). The sequence of this short DNA primer, 5'-GTAA-3', corresponds to an RNA sequence, 5'-UUAC-3', which is present both in the DR1 and bulge region of epsilon. While it was originally thought that reverse transcription initiated within the 5' DR1 sequence, studies later showed that the bulge sequence of epsilon provided the template for the incorporation of the first four nucleotides of (-) strand DNA (116, 160, 172). This finding was achieved by introducing mutations at the two potential initiation sites and examining the effects on the order of nucleotides incorporated onto the polymerase (172). The specificity of the site of initiation is not absolute as the polymerase can initiate reverse transcription from multiples sites within both the bulge and upper stem of epsilon (158). However, the polymerase does appear to prefer to initiate with a dGTP residue opposite a template C. The reason for this preference is unclear.

It is not fully understood why the polymerase halts synthesis at this point after only four nucleotides. Structural studies suggest that synthesis is limited to four nucleotides because the remaining two nucleotides present in the bulge are inaccessible for polymerisation (76). This may be due to either RNA-RNA interactions or to protein-RNA interactions. In addition, there is evidence that the following step in reverse transcription, transfer of the (-) strand DNA primer to the DR1 acceptor site at the 3' end of the pregenomic RNA, may be limited by the length and/or sequence of the primer. Indeed, insertion of additional nucleotides 5' to the UUAC template extends the length of the primer but the efficiency of the template switch is reduced. Limiting the primer to four nucleotides may be necessary to ensure both efficient dissociation from the RNA template and sufficient complementarity between the (-) DNA primer and the acceptor site present in DR1. It is interesting that telomerases, cellular reverse transcriptases which are responsible for the synthesis and maintenance of telomeres, also copy only a limited portion of RNA template followed by a template switch. It is speculated that, similar to DHBV polymerase, synthesis of DNA by telomerase is halted by the presence of a short RNA motif that engages in either an RNA-RNA or RNA-protein interaction that prevents further polymerisation of DNA (4, 97).

Priming of reverse transcription thus occurs in 2 steps: the attachment of dGMP via a phosphodiester bond to the hydroxyl group of a tyrosine followed by the additon of three more nucleotides using the bulge of epsilon as the template. These two steps appear to be distinct biochemical reactions. Evidence for this comes from the fact that certain reverse transcriptase inhibitors, such as PFA, have no effect on protein priming but block subsequent DNA synthesis (148, 173). In addition, polymerase variants of DHBV have been identified which are fully active for priming, the covalent attachment of the first dGMP to protein, but are defective in subsequent DNA elongation (142). Both the priming reaction and subsequent DNA elongation require the reverse transcriptase active site, the YXDD motif, which is conserved among viral reverse transcriptases. However, another region near the C-terminus of the reverse transcriptase domain appears to be required for continued DNA synthesis (174). It is speculated that the switch from protein priming to DNA elongation requires a conformational change in the polymerase (174).

Following priming, the DHBV polymerase and 4-nucleotide DNA complex dissociates from the RNA template and is transferred to the 3' end of the pgRNA where it anneals to the complementary sequence 5'-UUAC-3' at nucleotide 2534 within DR1 (101, 158, 172). It then serves as the primer for the synthesis of the (-) strand DNA which proceeds toward the 5' end of the pgRNA. Several factors appear to regulate this (-) strand DNA primer transfer. These include the attached polymerase protein, sequence complimentarity between the primer and the acceptor site, and position of the acceptor site. The transfer can be inhibited by mutation of amino acids 79-88 of the DHBV polymerase protein, indicating that these residues located in the middle of the terminal protein domain are important for the transfer (49). Sequence complementarity between the (-) strand DNA primer and the acceptor site in DR1 is preferred but it is not absolutely required for primer translocation (158). Some mispairing is tolerated as mutations within the acceptor site at DR1 do not dramatically decrease the levels of viral DNA synthesised, suggesting that (-) strand DNA transfer is not reduced. There is evidence that position of the acceptor site in relation to the (-) strand DNA primer is also involved in directing the specificity of the transfer (101). Since additional UUAC motifs present on the pregenomic RNA do not serve as acceptor sites, the UUAC *cis*-element cannot independently specify acceptor location. In addition, transfer to the wild-type acceptor site UUAC at position 2534 is preferred over adjacent UUAC motifs even when the wild-type acceptor position is mutated to create mispairing between acceptor site and (-) strand DNA primer (101). As well, when the wild-type acceptor site is disrupted by the insertion of additional nucleotides, transfer occurs to a new site within a few nucleotides of the wild-type position (101). This suggests that although the site of transfer is not invariable, it is limited to a small region in which a specific secondary structure may facilitate the transfer. Additional *cis*-acting elements required for strand transfer have been identified within the central domain of the pregenomic RNA but their function remains unclear (113). Although DR2 does not act as an acceptor site for (-) strand DNA primer translocation, it does appear to have some role in the synthesis of (-) strand DNA. Mutations in DR2 result in a reduction of (-) strand DNA and it is speculated that the primer transfer is inhibited, although the mechanism of the inhibition is not known (102).

Following transfer of the (-) strand DNA primer to the 3' terminus of the pregenomic RNA, (-) strand DNA synthesis proceeds using the RNA as template. Efficient elongation of the (-) strand DNA following the template switch requires the RNaseH activity of the polymerase protein, which removes the RNA strand of the RNA-DNA hybrid (19). The RNaseH activity is also responsible for generating the 18 nucleotide RNA primer that is used to initiate synthesis of the (+) strand DNA (100). This RNA primer contains the 12 nucleotide DR1 sequence at its 3' terminus.

1.3.11 Synthesis of (+) strand DNA.

Like synthesis of (-) strand DNA, synthesis of (+) strand DNA is discontinuous and involves both a primer transfer and a template switch. There are two pathways for (+) strand DNA synthesis, each producing a specific form of the viral genome. In the majority of cases, the 18 nucleotide RNA primer generated by RNaseH activity is translocated to the 5' end of the (-) strand DNA where it base-pairs to an acceptor site within DR2 (94). Here (+) strand DNA synthesis is initiated and proceeds to the 5' end of the (-) strand DNA to generate a (+) strand DNA primer of approximately 50 nucleotides. An additional template switch of this primer to an acceptor site within DR1 at the 3' end of the (-) strand DNA circularizes the genome, creating the relaxed circular form of the viral genome. Synthesis of the (+) strand DNA then proceeds to variable endpoints.

Approximately 10% of the time, the first primer translocation does not occur and synthesis of (+) strand DNA proceeds from the 3' end of the (-) strand DNA (147). This is known as *in situ* priming and results in the formation of the linear duplex form of the viral genome. The mechanism of discrimination for these two pathways was elucidated by studying DHBV variants with abnormally high levels of *in situ* priming. It was found that mutations in these variants disrupt a small DNA hairpin structure which is normally present in the 5'end of the 3' copy of DR1 of the (-) strand DNA (59). This hairpin regulates the RNA primer translocation by making the 3' end of the (-) strand DNA a poor template for the initiation of (+) strand DNA synthesis which, for the most part, effectively inhibits *in situ* priming.

Investigation of the mechanism of both primer translocation and circularisation of the genome during (+) strand DNA synthesis has revealed a complex process involving both host factors and multiple *cis*-acting sequences in the (-) strand DNA template. As with the translocation of (-) strand DNA primer, sequence complementarity between the RNA primer and the acceptor site within DR2 is important but not sufficient to direct primer translocation during (+) strand DNA synthesis. Mutations in either the donor site (DR1 of the (-) strand) or acceptor site within DR2 inhibit primer translocation (102, 147). However, when complementary mutations are introduced simultaneously into the two sites, primer translocation is not necessarily restored (102).

There are a number of *cis*-acting elements required for (+) strand DNA synthesis and circularisation of the genome. Besides the donor and acceptor sites present in the terminal redundancy at either end of the (-) strand DNA template, additional sequences have been identified at the 5', middle, and 3' regions of the (-) strand DNA template, termed 5E, M, and 3E, respectively (60, 112, 113). DHBV variants with mutations in these regions are partially defective for both primer translocation to DR2 and circularisation of the genome. By studying HHBV variants in which regions of the genome homologous to these three sites were replaced with DHBV sequence, it was determined that the 3 sites likely interact during (+) strand DNA synthesis, although how this occurs is not known (113). The interaction of these sites may organize the (-) strand DNA template to facilitate efficient template switches. An additional *cis*-acting element, termed upstream binding site (UBS), has been identified just 5' of the epsilon structure which interacts with a cellular factor, p65 (125, 126). Mutations in UBS prevent p65 binding which selectively inhibits (+) strand DNA synthesis (125). The mechanism of this inhibition remains unclear.

The last template switch and subsequent circularisation of the viral genome is sensitive to the position of the 5' end of the (+) strand relative to the site of the template switch (103). When the acceptor site for the RNA primer is moved to a more internal position on the (-) strand DNA template such that (+) strand DNA synthesis is initiated from a new position, no circularisation occurs.

1.3.12 Virus morphogenesis.

It appears that virion assembly and export from the cell is initiated when nucleocapsids bud into the ER to acquire surface antigen-containing envelopes. The virion is then exported via the constitutive secretory pathway. Morphogenesis occurs in three disctinct steps: the interaction of the nucleocapsid with the L surface antigen present in the ER membrane, budding of the nucleocapsid into the ER lumen and transport of the enveloped virion out of the cell via the Golgi apparatus.

There are two lines of evidence that the nucleocapsid- L surface antigen interaction occurs at the ER membrane rather than the plasma membrane. First, immunostaining and electron microscopy indicate that the majority of HBV surface antigen is present in intracellular membranes rather than the plasma membrane (122). Second, double immunofluorescence was used to co-localize HBV L surface antigen with resident ER proteins such as protein disulfide isomerase (71). This would make it unlikely that the nucleocapsids obtain their envelope by budding from the plasma membrane, where only trace amounts of surface antigen are present.

There is evidence that virion envelopement is linked to viral genomic DNA maturation, most likely at the level of nucleocapsid interaction with L surface antigen. Serum virions contain predominantly mature genomes while capsids from intracellular virus contain a mixture of genomes from various stages of DNA synthesis (110, 150). In addition, nucleocapsids produced from both HBV and DHBV genomes which contain mutations in the polymerase active site are not enveloped or secreted (48, 176). These polymerase mutants are defective for all DNA synthesis but still allow efficient pregenomic RNA encapsidation. Analysis of additional polymerase mutants indicate that (-) strand and preferably some (+) strand DNA synthesis must occur before nucleocapsids can be selected for envelopement (176). These results suggest that nucleocapsids containing mature DNA genomes relay some sort of signal on their surface which allows them to interact with surface antigen, facilitating their envelopement.

There is evidence to suggest that a change in the level of core protein phosphorylation may play a role in the signalling process. The core protein from intracellular capsids is hyperphosphorylated, as evidenced by its heterogeneity on SDS-PAGE which can be removed by alkaline phosphatase treatment of native cores (130, 136). Core protein from serum-derived virions does not display this heterogeneity (130). Selective membrane attachment of intracellular nucleocapsids containing de-phosphorylated core protein and mature DNA genomes has been demonstrated (106). However, phosphatase treatment of hyperphosphorylated capsids did not confer membrane-binding ability, indicating that de-phosphorylation alone is not sufficient to induce membrane binding. Suprisingly, this selective membrane attachment of dephosphorylated capsids is independent of the presence of the L surface antigen (106). It does appear that the large surface antigen contributes to the stability of the membrane-capsid interaction, as indicated by a reduction of membrane-associated capsids in the absence of L surface antigen (106). The current model for virus morphogenesis therefore, as proposed by Mabit *et al* , involves the synthesis of a mature DNA genome within cytoplasmic nucleocapsids which are initially hyperphosphorylated and unable to bind cellular membranes. Genome maturation induces a change in the surface of the capsid structure which facilitates membrane association. The L surface antigen then interacts with membrane-bound capsids, leading to budding of the capsids into the ER lumen followed by secretion of enveloped virions. A vast excess of SVPs containing both large and small surface antigen are also secreted with the virions.

1.4 Viral gene products.

A striking feature of hepadnaviruses is the multi-functional nature of the viral proteins. All of the viral antigens, which include e antigen, core protein, the two surface antigens and the polymerase protein, have numerous functions in the life cycle of the virus.

1.4.1 Precore protein (e antigen).

All hepadnaviruses express a secreted form of the core protein called e antigen. This protein is expressed using the core gene plus an additional in-phase upstream region termed the Pre-core region. As already discussed, biosynthesis of this protein involves both N and C-terminal processing in the secretory pathway of the cell and export as a soluble protein. The role of this protein in hepadnaviral infections is not clear. DHBV PreC-AUG variants, which do not express e antigen, are able to establish viremia in ducklings with no apparent differences in either viral protein expression or virus secretion kinetics (137, 139). Futhermore, these mutant genomes do not revert to wild-type even after several passages in ducks. The genetic stability of these mutants would seem to suggest that e antigen does not play an essential role in the virus life cycle. However, the fact that e antigen expression is conserved among hepadnaviruses, a notoriously frugal virus when it comes to genome organisation, questions this conclusion. The role of e antigen may be in establishing or maintaining a persistent infection, something that was not investigated in the duck studies. Persistent infection might be achieved by modulating either the immune response or the viral replication levels. There is some evidence to suggest that e antigen negatively regulates viral replication levels. First, HBV pre-core mutants, which do not express e antigen, have been associated with higher viral replication levels (88). Second, *in vitro* studies using cloned HBV variants demonstrated that e antigen expression inhibited HBV replication, possibly at the level of transcription (88). This is also consistent with the observation that a HBV precore protein derivative is translocated into the nucleus (121). Third, it appears that the levels of e antigen are relatively low as synthesis of the precore mRNA transcript is negatively regulated by a *cis*-acting sequence. This would make sense if indeed e antigen negatively regulates hepadnaviral replication.

1.4.2 Core protein.

The core protein plays both a structural and functional role in the replication of hepadnaviruses. The structural role involves the formation of the viral nucleocapsid. The icosahedral viral nucleocapsid is comprised of 180 core protein subunits which have the capacity to self-assemble in the absence of other viral proteins. Core protein also plays a role in RNA encapsidation, viral DNA synthesis and cellular trafficking of mature nucleocapsids.

The core protein is comprised of two separate functional domains. The N-terminal region (amino acids 3-66) is important for the tertiary structure of the core protein. A series of insertion and deletion mutants have shown that the N-terminal and central regions (amimo acids 3-228) are important for the self-assembly of core particles in *E.coli* (177). The C-terminal domain (amino acids 181-228) contains an arginine-rich region which possesses a non-specific DNA–binding activity. The C-terminal domain is required for viral DNA synthesis as mutations in this region or removal of the C-terminal 36 amino acids results in core particles that are defective in genome replication (136, 178). These mutants remain competent for pregenomic RNA packaging, however, indicating that this domain is not involved in RNA encapsidation (136, 178).

Phosphorylation/dephosphorylation of core protein appears to regulate multiple steps in the DHBV replication cycle, including RNA packaging, DNA synthesis and targeting of nucleocapsids to various cellular compartments. As mentioned earlier, core protein is phosphorylated at multiple sites. Removal of the last 36 C-terminal amino acids eliminates the extensive phosphorylation associated with core protein, indicating that the majority of the phosphorylation sites are within this serine-rich region (136). Site-directed mutagenesis indicated that this region contains four phosphorylation sites (T239, S245, S257, S259) (180). In addition, core protein may be phosphorylated at T174 (5, 180).

Phosphorylation of DHBV core protein appears to play only a minor role in the packaging of pregenomic RNA. Deletion of either the 12 or 36 C-terminal residues, where the majority of the phosphorylation occurs, did not affect the ability of core to package pregenomic RNA (136). This is in agreement with a separate study, where RNA packaging did not require any specific phosphorylation state of the C-terminal serine/threonine residues of core (179). When these residues are all changed to alanine, there is a moderate effect on the ability of core to package RNA (46). It is speculated that phosphorylation of the C-terminal threonine/serine residues optimises but is not essential for RNA encapsidation. However, another study demonstrated that phosphorylation of a separate domain of core does appear to regulate packaging of pregenomic RNA by the core protein. RNA packaging was found to be reduced 100-fold when the threonine 174 of a potential threonine-proline kinase recognition motif (recongnition sites for CDK and MAPK kinases) in the core protein was changed to alanine, indicating that phosphorylation of this residue is required for efficient packaging (5). Unlike the studies examining the effects of phosphorylation at residues in the C-terminus of core, the presence of phosphorylation at this threonine 174 has not been demonstrated directly. However, the motif does contain a down-stream proline which has been shown to be essential for the phosphorylation of serine and threonine residues in core (180). The same study also demonstrated the presence of one or more sites of phosphorylation at an internal position of the core protein.

Different patterns of phosphorylation of the C-terminal serine residues appears to play a role in viral DNA synthesis. Yu *et al* examined the role of phosphorylation in viral DNA synthesis by substituting serine residues with either alanine or aspartic acid to mimic either serine or phosphoserine, respectively (179). The presence of phosphorylation at postitions S245 and S259 is required for generation of mature relaxed circular DNA within the nucleocapsids. More specifically, P-S259 is required for synthesis of the (-) strand and P-S245 is required for the synthesis of the (+) strand. It is possible that these positions interact with the various *cis*-acting elements required during both (-) and (+) strand DNA synthesis. The presence of phosphorylation at S257 inhibits cccDNA synthesis, but not (+) and (-) strand DNA synthesis, possibly by preventing the re-cycling of newly synthesised mature nucleocapsids back to the nucleus. The absence of phosphorylation at ser 257/259 stimulates the production of extracellular virus. The de-phosphorylation of S257 and S259 may be the signal that the viral DNA synthesis is complete and that the nucleocapsid is ready for envelopement by the L surface antigen. In addition, phosphorylation of S259 is required for DHBV infection of PDHs (179). It is speculated that phosphorylation at this site might stimulate dissociation of the nucleocapsid in addition to stimulating the synthesis of (-) strand DNA. Yu *et al* propose that this apparent sequential modification of core protein by phosphorylation and de-phosphorylation of specific residues is required for the nucleocapsid to proceed through DNA synthesis and virus assembly, perhaps by stabilizing different conformations of core protein (179). There is some evidence that specific conformations of the C-terminus are associated with different combinations of phosphorylated serines (180).

In addition to the role in pregenomic RNA packaging and DNA synthesis, phosphorylation of core protein may play a key role in the cellular trafficking of cytoplasmic nucleocapsids. Immature nucleocapsids containing pregenomic RNA and (-) strand DNA are localised to the cytoplasm wherease nucleocapsids present in extracellular virions contain only the completed viral genome (both (-) and (+) DNA strands). Nucleocapsids with complete viral genomes must therefore display a signal that targets them for packaging (by the envelope protein) and export from the cell. It is possible that the signal is the de-phosphorylation of core protein. Core proteins from cytoplasmic capsids containing immature DNA were found to be phosphorylated, resulting in core protein heterogeneity on SDS-PAGE, (130). There appears to be some controversy over the position of the phosphorylation. In this study, the phosphorylation was present on the exterior of the capsid, as it was removed by phosphatase treatment of native capsids (130). However, a separate study indicated that the phosphorylation was not present on the surface of the capsids, as demonstrated by the resistance of the phosphate groups to phosphatase treatment unless the capsids were first denatured (136). Contrary to immature nucleocapsids, the core protein from extracellular virions, which contain only mature viral DNA genomes, appears to be unphosphorylated (130). This indicates that lack of phosphorylation of core might be the signal that directs capsids containing mature genomes to be packaged and exported from the cell. Further study showed that approximately one-half of cytoplasmic nucleocapsids are membrane associated and that this population contains predominantly mature, double-stranded DNA genomes and lack core phosphorylation (106). This would seem to suggest that the phosphorylation state of core does play a role in directing mature nucleocapsids for packaging and export. However, it was also discovered that removal of surface-exposed phosphates from capsids did not confer membrane affinity. De-phosphorylation of core, although correlating with membrane attachment, is not sufficient to confer membrane affinity to free nucleocapsids.

Mabit *et al* proposed a model where maturation of viral genomes proceeeds in nucleocapsids which are initially phosphorylated and incapable of binding to cellular membranes (106). The maturation of the genome induces a change in the capsid structure which triggers membrane association, possibly through exposure of L surface antigen binding sites, facilitating packaging of mature nucleocapsids. This is either preceeded or followed by de-phosphorylation of core protein. Selective membrane attachment of mature nucleocapsids does require the presence of the L surface antigen. The envelope protein appears to contribute to the stability of the membrane binding as the amount of membrane-bound capsids is decreased in the absence of the L envelope protein.

These results indicate that complex patterns of phosphorylation are required for core protein to perform its many functions. In general, phosphorylation of core appears to be required for functions carried out by immature nucleocapsids, while it inhibits the functions of mature nucleocapsids. It is important to note that most studies examining the role of phosphorylation involve the substitution of the amino acid of interest with either alanine or aspartic acid, with the assumption that these changes mimic unphosphorylated or phosphorylated residues, respectively. However, the possibility of effects caused by these substitutions that are independent of effects caused by phosphorylation cannot be ruled out. The identity of the kinase responsible for the phosphorylation of DHBV core protein has not been determined. However, a recent study has identified SR protein-specific kinase 1 and 2 (SRPK1 and SRPK2) as the major cellular kinase responsible for the phosphorylation of HBV core (35).

1.4.3 Polymerase protein.

The polymerase protein serves multiple vital functions in the replication of DHBV, including the inititation of pregenomic RNA encapsidation, assembly of nucleocapsids, priming of reverse transcription and synthesis of both (-) and (+) DNA strands. To perform these various functions, the polymerase protein is organised into separate domains: the terminal protein, spacer region, reverse transcriptase and RNase H domains. In addition to having separate domains for the various functions, the polymerase protein undergoes several conformational changes to regulate its enzymatic activites.

Early studies demonstrated that hepadnaviral genomes containing mutations in the polymerase gene synthesise capsids that lack viral RNA, indicating that the polymerase protein is required for encapsidation of the pregenomic RNA (6, 62). It was later shown that the pregenomic RNA encapsidation is initiated when the polymerase protein interacts with the packaging signal, epsilon, present at the 5' end of the pgRNA (128). This then presumably facilitates the interaction of the polymerase protein-RNA complex with core dimer subunits, leading to the assembly of the nucleocapsid. It is not clear which region of the polymerase protein is responsible for the encapsidation function. Studies with a series of frameshift mutations indicated that either encapsidation requires multiple regions of the protein or that the function maps to the C-terminus of polymerase protein (19). The encapsidation process requires the polymerase protein to interact with both epsilon sequences, present on the pregenomic RNA, and the core dimer subunits and it is possible that these two interactions map to separate regions of the polymerase.

The terminal protein domain is located at the N-terminus of the polymerase protein and includes amino acids 1-220 (174). The function associated with this region is the priming of reverse transcription (12, 171, 174, 175). Immunoprecipitation studies using anti-polymerase antibodies indicated that the polymerase protein is covalently bound to the 5' end of the (-) strand DNA (12). It was later discovered that this was due to the fact that the polymerase primes synthesis of (-) strand DNA using the bulge sequence of epsilon as the RNA template (171). The priming reaction involves the formation of a covalent phosphodiester bond between tyrosine 96 of the polymerase protein and dGMP, the first nucleotide of the (-) strand DNA (175, 186). Mutational analysis indicates that this tyrosine is essential for priming of reverse transcription.

Unique to hepadnaviral reverse transcriptases is a domain referred to as the spacer domain, located between amino acids 220-350 (174). The sequence of this region of the polymerase is poorly conserved and its function remains unclear. The various functions of the polymerase are unaffected by large insertions into this domain and it is speculated that the spacer domain acts as a hinge region which separates the priming and polymerisation activity domains of the polymerase protein (19).

The reverse transcriptase domain is located between amino acid 350-650 of the polymerase protein (174). This domain has both RNA and DNA-dependent DNA polymerase activity and is responsible for the synthesis of the hepadnaviral genome. The hepadnaviral reverse transcriptase domain has sequence homology with reverse transcriptases of retroviruses, including the active site of the enzyme known as the YMDD (tyrosine-methionine-aspartic acid-aspartic acid) motif (19, 161). The polymerase activity is restricted to the DHBV template and is unable to act on exogenous templates (133). This may be due to the fact that the reverse transcriptase activity is dependent on the association of the polymerase protein with epsilon. The continued interaction of polymerase with the DHBV genome may be required for the maintenance of this activity.

The polymerase activity is an obvious target for antiviral therapy and the use of nucleoside analogues have been successful as anti-hepatitis B agents. Lamivudine, or 3TC, is a potent inhibitor of hepadnaviral polymerase/reverse transcriptase activity and its incorporation results in chain termination during hepadnaviral DNA synthesis (143). It has been shown to be extremely effective in reducing HBV DNA levels and improving liver histology in chronic HBV- infected individuals (37, 84, 85, 127). Other nucleoside analogues, such as penciclovir and carbocyclic 2'-deoxyguanosine (CDG), target the priming and reverse transcriptase activities of the polymerase. The first nucleotide incorporated into the (-) DNA strand is a dGMP and so these nucleotides, as guanosine analogues, are capable of inhibiting the synthesis of the (-) strand DNA primer. Other antiviral compounds which target the polymerase include pyrophosphate analogues, phosphonoformic acid (PFA), which inhibit (-) strand DNA elongation (148, 173). Due to the emergence of drug-resistant variants, long-term successful antiviral therapy will likely involve combination therapy using compounds which target a variety of polymerase activities as well as other steps in the viral replication.

Unique to the hepadnaviral reverse transcriptases is an additional enzymatic activity known as pyrophosphorolysis where the byproduct of DNA polymerization, pyrophosphate (PPi), serves as the substrate for the reverse reaction which results in the removal of newly incorporated nucleotides from the DNA chain. It has been shown that the concentration of PPi within the replicating cores reaches a concentration sufficient to inhibit viral DNA synthesis (167). It is speculated that this reaction may partially account for the apparent slow rate of DHBV DNA replication. Also, it may explain why certain nucleoside analogues, such as lamivudine, are effective inhibitors of DHBV replication while others, such as ddC, are not. Lamivudine has been shown to be resistant to removal by pyrophosphorylisis whereas ineffective inhibitors, such as ddC, are readily removed by pyrophosphorylysis.

The RNase H domain is located at the C-terminus of the polymerase protein and includes amino acids 650-786 (174). This enzymatic activity is responsible for the degradation of RNA in the RNA:DNA hybrid created by reverse transcription of the pregenomic RNA. It contains highly conserved sequences analogous to other known RNase H enzymes, including RNase H proteins from HIV, RSV, MoMLV and HBV (19). The RNase H activity is unable to act on exogenous DNA:RNA heteroduplexes (50). Mutation of either glutamic acid 696 or apartic acid 715, both within the catalytic site of the RNase H domain, inhibits (+) strand DNA synthesis, as expected (26). Unexpectedly, these mutants are capable of initiating (-) strand DNA synthesis normally but they exhibit inefficient elongation of the (-) strand (26). It appears that the polymerase pauses at specific locations on the pregenomic RNA, although the reason for this is not clear. Interestingly, mutation of two other residues thought to make up the RNase H catalytic site, asp-666 and asp-755, inhibits synthesis of both (-) and (+) DNA strands, as indicated by endogenous polymerase assays and Southern blot analysis of nucleic acids from cytoplasmic cores (19).

In addition to the RNase H activity required for viral DNA synthesis, the RNase H domain of the polymerase appears to have a role in encapsidation of the pregenomic RNA. A DHBV variant with a mutation of cysteine 711 of the polymerase protein, within the RNase H domain, was found to be defective for viral RNA encapsidation (27, 28). Additional mutagenesis studies indicated that the basic structure of the RNaseH domain is required for RNA packaging (28). These packaging-defective mutants retained the ability to prime (-) strand DNA synthesis, although at reduced (5-20 fold) levels (28). A separate study using C-terminal truncations of the polymerase protein demonstrated that amino acid residues beyond position 568 are not required for either RNA binding or priming activity (128). The RNase H domain's role in encapsidation may therefore be to interact with the core protein rather than epsilon. This would explain why the RNase H mutants defective for packaging RNA are still competent for priming (-) strand DNA synthesis.

In addition to the multiple functional domains, the polymerase protein appears to undergo several conformational changes as it proceeds from one activity to the next, as summarised in Wang *et al* (174). The first conformational change occurs when polymerase interacts with the cellular chaperone protein Hsp90 complex. This "activates" polymerase to an epsilon-binding competent form. Binding to epsilon then intitiates the second conformation change which may be required for the enzymatic activity of the polymerase. Following the polymerase-primed addition of the first dGMP, an additional conformational change may be required for the polymerase to switch from a priming to an elongation mode to allow further synthesis of the (-) strand primer. A final conformation change occurs when the polymerase–primer complex dissociates from the 5'end of the pregenomic RNA and translocates to the 3' end of the RNA to continue synthesis of the (-) strand DNA. The flexibility of the polymerase protein, indicated by multiple conformational changes, is thus a mechanism to regulate the various enzymatic activities.

1.4.4 L and S surface antigens.

The DHBV genome encodes two envelope proteins, the S and L surface antigens. They are encoded by the same open reading frame but are expressed from separate mRNA transcripts (16). The L surface antigen is comprised of the 162 amino acids of the S surface antigen plus an additional 162 amino acids at the N-terminus which are encoded by the PreS region of the envelope open reading frame. The L surface antigen is myristilated on glycine residue 2 and this modification is essential for the infectivity of the virus particle (107). Myristilation also appears to be required for proper assembly and secretion of L surface antigen. The DHBV S and L surface antigens are not glycosylated, unlike the HBV envelope proteins, despite the presence of at least two potential N-glycosylation sites (47).

The envelope proteins, particularly the L surface antigen, have several essential functions in the replication cycle of DHBV. Both envelope proteins serve structural roles, as the major component of the virion envelope and, in the case of L surface antigen, as a matrix protein. The PreS domain of the L surface antigen is responsible for the attachment of the virion to hepatocytes via interaction with CPD, as already discussed in section 1.3.1. The PreS domain also regulates the amplification of cccDNA, which serves as the template for all further viral replication. There is preliminary evidence which suggests that a phosphorylated form of the L surface antigen can function as a transcriptional activator.

The envelope proteins make up the major componant of the viral envelope. Both the S and the L surface antigens are essential for virus assembly and secretion (152). In the absence of the S surface protein there is no secretion of enveloped virus (152). This may be explained by the observation that, in the absence of S protein, the L surface antigen is intracellularly retained (47). The L surface antigen also serves as a matrix protein in that the cytosolic PreS domain interacts with mature nucleocapsids to facilitate virus assembly and export through the ER and Golgi apparatus (155).

The L surface protein serves as a negative regulator of cccDNA amplification (91, 151, 152). cccDNA is initially formed by the conversion of the rcDNA from the original infecting virus into cccDNA. Amplification of this single cccDNA molecule then occurs via an intracellular pathway involving transcription of the pregenomic RNA, packing of the pregenomic RNA into nucleocapsids in the cytoplasm, synthesis of rcDNA and transport of this newly synthesised rcDNA into the nucleus where it is converted to cccDNA (164). For the most part, amplification of cccDNA molecules occurs in the early stages of DHBV infection and proceeds until a pool of 20-30 copies of cccDNA/nucleus is produced. Further amplification is then inhibited by the presence of the L surface protein (91, 151, 152). The level of viral protein expression early in infection is low due to the low copy number of cccDNA molecules, which serves as the template for viral gene expression. As the pool of cccDNA increases, so does the amount of viral protein present in the cell. Initially, there are low levels of envelope proteins present and so the newly synthesized nucleocapsids are diverted to the nucleus where the rcDNA is converted to cccDNA. Once a threshold level of L surface antigen is present, the nucleocapsids interact with the cytosolic PreS domain of L surface antigen which facilitates virus assembly and export. Envelope-deficient DHBV mutants accumulate higher levels of cccDNA compared to wild-type because the nuclear import of nucleocapsids is not inhibited by the presence of L surface antigen. Analysis of L surface antigen mutants with linker-encoded amino acid substitutions revealed, not surprisingly, that the functions of cccDNA regulation and virus assembly/secretion map to the same domain of PreS on the L surface antigen (91). Co-transfection studies using an envelope-deficient DHBV genome and various mutant L surface antigen constructs revealed that substitutions between amino acids 117 and 136 result in both a high accumulation of cccDNA and a lack of production of enveloped virus. The two functions of cccDNA regulation and virus assembly/secretion are therefore intrinsically linked. Further analysis using single amino acid substitutions showed that amino acids D128 and L131 were involved in both functions. It is possible that this region of the PreS domain is responsible for the interaction of L surface antigen with mature nucleocapsids.

Regulation of the cccDNA pool may be key to the virus' ability to establish a persistent infection in the absence of any apparent cytopathogenicity. Envelope-deficient DHBV is unable to maintain an infection in PDHs, with virus production decreasing rapidly after 12 days post-infection (151). DHBV mutants with mutations in L surface antigen which cause increased accumulation of cccDNA have been shown to be cytopathic both *in vivo* and *in vitro* (90). Interestingly, abnormal expression or localisation of L surface antigen in HBV infection is sometimes associated with cytopathic effects. If higher levels of L surface antigen are indeed cytopathic, then it is logical to conclude that it is the levels of this specific viral protein which are responsible for negatively regulating the pool of cccDNA and hence level of viral replication.

In addition to the essential functions mentioned above, the L surface antigen may have an additional regulatory role in DHBV infection. The cytosolic PreS domain has been shown to be specifically phosphorylated at serine 118 by extracellular signal-regulated (ERK)-type mitogen-activated protein kinase (MAP) (135). The amount of phosphorylated L surface antigen is barely detectable under physiologic conditions in the liver. However, the level of L surface antigen phosphorylation can be enhanced *in vitro* by a variety of specific stimuli, including low temperature, UV irradiation, or mitogenic signals which also increase ERK-type MAP kinase activity. Interestingly, the phosphorylation of L surface antigen correlates with its ability to activate gene expression. Although it is unlikely that the L surface antigen would be released from the ER membrane, it is possible that the phosphorylated L protein may initiate signalling via the Ras-Raf-MAP cascade from its cytoplasmic location by interacting with cytoplasmic proteins. Mutations in L surface antigen that eliminate or mimic phosphorylation at serine 118 do not significantly influence viral replication. These mutants also retain infectivity both in PDH cultures and in ducklings (53). This does not rule out the possibility that phosphorylation of L surface antigen may be a mechanism by which the virus can modulate replication in response to extracellular enviromental signals and hence maintain a persistent infection. The identification of cellular genes which are activated by the phosphorylated form of L surface antigen should facilitate greater understanding of the role of this protein in DHBV infection.

Like all hepadnaviruses, the DHBV envelope proteins are not only present in the envelopes of virus particles, they also assemble into SVPs which are secreted from infected cells in 100-1000 fold excess of virions. The vast excess of both envelope proteins produced during an infection in the form of SVPs is common to all hepadnaviruses and so likely perfoms an important function during infection. One role of these SVPs is possibly to act as an immune decoy for any anti-L surface antigen antibodies which may be produced during infection. In addition, they may play a role in viral morphogenesis by facilitating efficient transport of virions through the Golgi apparatus. L surface antigen expression has recently been shown to be capable of down-regulating the cellular receptor, CPD, in DHBV-infected cells (14). L surface antigen interacts with CPD and causes it to be retained in the ER. This prevents processing of the gp170 precursor protein, an incompletely glycosylated form of CPD, and results in its degradation. This intracellular CPD-L surface antigen interaction may be a mechanism to deal with a problem specifically encountered by enveloped viruses and may partially account for the vast excess of L surface antigen produced in DHBV infected cells. The viral envelope (glyco)proteins use the same secretory pathway for synthesis, maturation and export as do the cellular receptors utilized for virus attachment to cells. Consequently, the intracellular interaction of the viral envelope proteins with receptors could potentially interfere with virus morphogenesis. This is perhaps even more relevant in the case of hepadnaviruses, where the cytoplasmic capsids bud into a post-ER, pre-Golgi compartment rather than from the plasma membrane. Down-regulation of CPD was observed even when CPD over-expressed in DHBV-infected liver cells, indicating that L surface antigen expression is sufficient to interfere with CPD overexpression (14). The vast production of SVPs, which contain L surface antigen, may therefore be a means of clearing the cellular secretory pathway of receptor protein, allowing efficient virus export from the cell.

The removal of the DHBV receptor, CPD, from the cell surface of infected cells could also ensure that progeny virus selectively infects uninfected cells, allowing efficient spread of the virus throughout the liver. This is a mechanism of superinfection exclusion that is used by a number of viruses and will be discussed in greater detail in Chapter 4.

The L surface antigen, in particular the PreS domain, thus performs multiple specific functions in the replication of hepadnaviruses. The regulation of cccDNA amplification, selection of mature nucleocapsids for virus assembly, and potential transactivator functions of L surface antigen are dependent on the PreS domain being in a cytosolic/internal position. The PreS domain is also required to be on the surface of the virion, which is equivalent to a luminal position intracellularly, to function in the attachment of the virus to hepatocytes. To achieve these various roles, hepadnaviruses have adapted a unique strategy whereby L surface antigen forms mixed transmembrane topologies. The models of the various L surface antigen topologies are depicted in Figure 1.6. Protease sensitivity assays coupled with Western blots have indicated that approximately half of the L surface antigens in DHBV virions contain the PreS domain

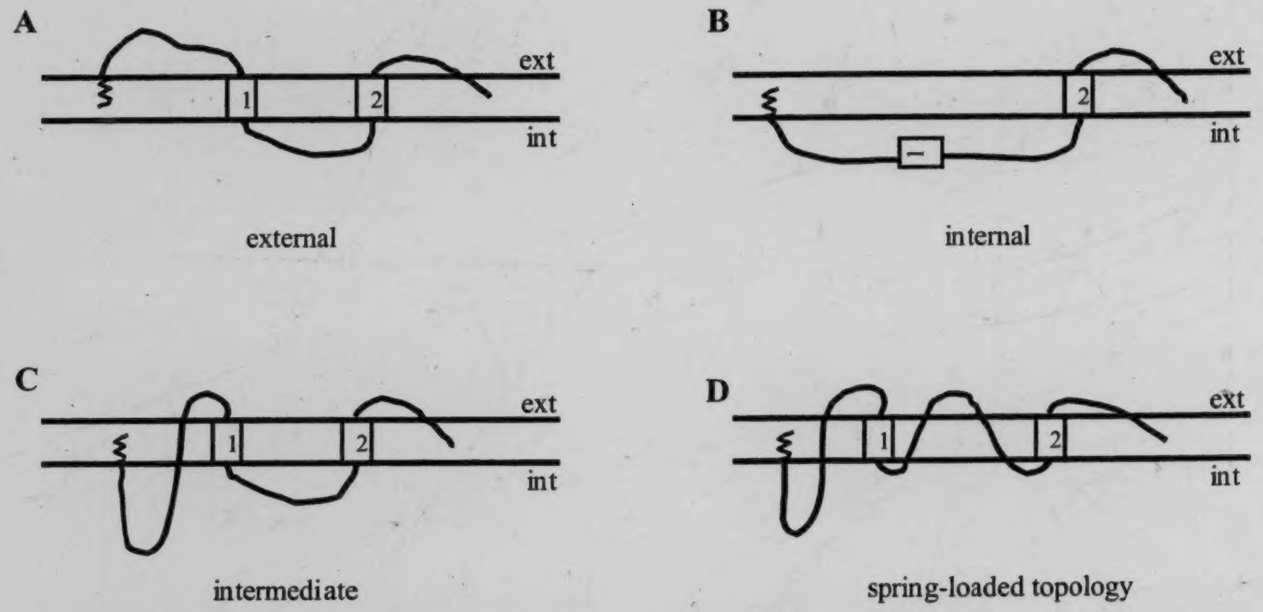

A
ext
1
2
int
external
B
ext
2
int
internal
C
ext
1
2
int
intermediate
D
ext
1
2
int
spring-loaded topology

Figure 1.4: Proposed models of DHBV large surface antigen topology (adapted from Grgacic et al, 2000). The transmembrane domains are indicated by boxes 1 and 2, respectively. The N-terminal myristate is represented by the spiral. ext., external; int., internal.

on the surface of the virion and half in an internal position (155). The internal topology of L surface protein has the transmembrane (TM) 2 domain of the S region traversing the membrane while the remainder of the S domain, including the TM1, and the entire PreS domain are cytosolic (Figure 1.3b) (54, 155). This topology is present immediately after synthesis (155). The external topology differs in that the the TM1 traverses the membrane and the entire PreS domain is translocated into the lumen of the ER, placing it on the surface of the virion following morphogenesis (Figure 1.3a) (57, 155). There is also evidence for an intermediate topology present in mature particles where the TM1 is inserted into the membrane while the PreS domain remains in the cytoplasm with the exception of a small region at the C-terminus (Figure 1.3c)(52, 54). The position of the C-terminus of the S domain of the S and L surface proteins has not been investigated but most models place it within the membrane (52, 54, 155). The N-terminus of the L surface antigen is also generally placed within the membrane (52, 54). Immunoprecipitation studies using SVPs and epitope-specific antibodies have suggested that a fraction of the L surface antigen also exists in a highly folded "spring-loaded" topology (Figure 1.3d) (52). In this topology, the normally cytoplasmically-located region between TM1 and TM2 traverses the membrane, creating two additional transmembrane regions, with the top of the loop protruding on the virion surface (52).

The mixed topology is the result of a partial postranslational translocation of the PreS domain. The mechanisms controlling PreS translocation are not fully understood. Initially, the PreS domain and the adjacent TM1 remain in the cytoplasm after synthesis but at some point fractions of the PreS domains are translocated into the lumen of the endoplasmic reticulum (155). There is evidence that co-translational translocation of the PreS domain is delayed by both a cluster of positively charged amino acids in the C-terminus of the PreS domain, adjacent to the TM1, and additional elements present in the central part of the PreS domain (155). Budding of the nucleocapsids into the lumen of the ER presumably occurs before all PreS domains are translocated, resulting in the mixed topology observed in virus particles. Translocation of the hydrophillic PreS domain across the plasma membrane does not occur in the absence of the S protein and it is speculated that S protein may form a channel through interactions between the amphipathic TM regions in oligomerised S domains (52).

Studies have shown that low pH treatment of DHBV particles induces a major conformational change in the S domain of L surface antigen as indicated by the exposure of an additional trypsin cleavage site (54). Western blot analysis using epitope specific antibodies mapped the potential cleavage site to two lysine residues (K204/206) in the region between TM1 and TM2, which is normally embedded in the membrane (54). At the same time, a cluster of lysines in the C-terminus of the PreS domain, adjacent to the TM1, is no longer susceptible to trypsin cleavage (54). The proposed conformational change involves translocation and exposure of the TM1 and at least part of the loop. It is unclear what happens to the PreS domain; it may become membrane embedded due to the obscuring of the trypsin cleavage site within this region. It is interesting to note that a putative fusion peptide, which closely matches a consensus sequence of fusion peptides present in a number of other viral glycoproteins (including HBV, Ebola, and HIV) (104), is present in the N-terminus of the S domain and partially overlaps with the TM1. The proposed conformational change may expose this fusion peptide, facilitating membrane fusion and viral entry. Indeed, the conformation change is associated with increased surface hydrophobicity, as indicated by viral particle aggregation and binding to liposomes (54). Studies using mutants of the putative fusion peptide may provide some insight into the role of this conformational change in DHBV entry.

Currently, the role of this proposed form of L surface antigen in DHBV infection, if any, is not clear. The conditions used in the study to induce the conformational change, low pH and reducing agents, are not physiologically relevant so are unlikely to be the natural inducer. Furthermore, binding of L surface protein to the DHBV cellular receptor, CPD, did not induce the change (54). Although low pH is unlikely to be the inducer of the conformation change, as DHBV entry is generally thought to be pH-independent, moderately low pH might effect the kinetics of entry. Indeed, the conformational change does occur at pH 5, which the virus may encounter in the early endosome (54). Interestingly, the change was shown to take a substantial period of time at this pH, approximatly 16 hours, which is consistent with the extended time required for complete DHBV entry into cells. Similar to the role of phosphorylation in core protein function, the multiple topologies of the L surface antigen is yet another example of hepadnaviruses remarkable ability to utlilize a single protein for several functions.

Thesis Goals:

A. The treatment of chronic HBV infection with the nucleoside analogue lamivudine results in the selection of lamivudine-resistant HBV. The first goal of this thesis was to develop a convenient *in vitro* assay to identify antiviral compounds active against lamivudine resistant HBV variants.

B. The lamivudine-resistant HBV variants appear to establish an infection more readily in an uninfected liver as opposed to an HBV-infected liver as evidenced by the more frequent and rapid emergence of lamivudine-resistant HBV in transplant patients compared with non-transplant patients. One possible explanation for this observation is a phenomenom called superinfection exclusion where a cell infected with a virus is resistant to superinfection by the same virus. The second goal of this thesis was to use the DHBV animal model to investigate whether superinfection exclusion occurs in hepadnaviral infection.

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Chapter 2

Generation of Stable Cell Lines Expressing Lamivudine-Resistant Hepatitis B Virus for Antiviral-Compound Screening. [1]

2.1 Introduction.

Over 350 million people worldwide are chronically infected with HBV despite the availability of an effective vaccine. Chronic HBV infection is associated with both cirrhosis and hepatocellular carcinoma (5, 6, 35). Until recently, the only licensed treatment available was interferon-alpha (IFN-α) which has a response rate of only 30-40% (56). The (-) enantiomer of 2'-dideoxy-3'-thiacytidine, also known as lamivudine, was shown to be a potent inhibitor of HBV replication (12, 13, 17, 45) and was liscensed in Canada in 1998. Lamivudine contains a sulfur instead of a carbon at the 3' position and lacks the 3' OH group necessary for chain elongation during DNA replication (Figure 2.1). It competes with cellular dCTP and is incorporated by the viral polymerase, causing chain termination during both the reverse transcription and DNA synthesis steps of HBV-DNA synthesis (49).

Chronically infected individuals treated with long-term lamivudine therapy show a significant reduction of viral load and histological improvement (16, 29, 30). Lamivudine has also been shown to be beneficial in both the prevention and treatment of re-infection of livers transplanted into HBV carriers (4, 8, 9, 21, 40). A drawback to lamivudine therapy is that the template for hepadnavirus replication, the established cccDNA pool, is only slightly affected by lamivudine (39). The cccDNA exists as a pool of 20-30 molecules per diploid genome and is established early in infection (1). Although antiviral therapy should prevent, or at least severely reduce, the formation of new cccDNA molecules in an infected hepatocyte, elimination of HBV infection requires the complete elimination of cccDNA. Studies have shown that cccDNA is stably associated with protein in the form of a minichromosome (41). Viremia in DHBV-

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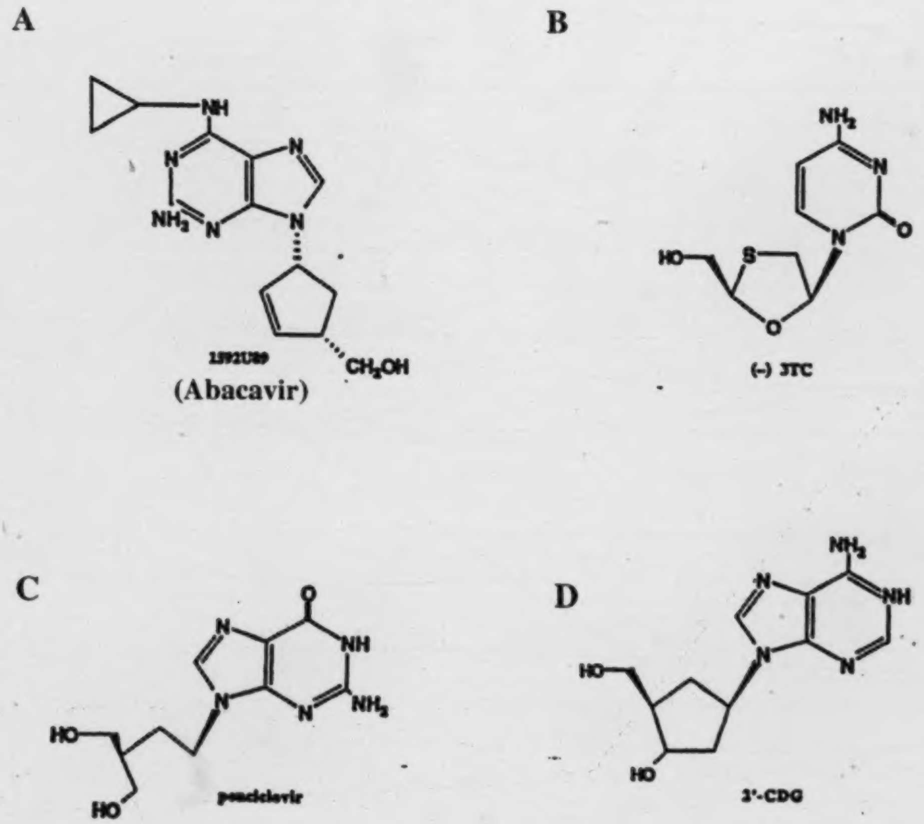
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Figure 2.1: Chemical structures of nucleoside analogues.

infected ducks which had been suppressed with continuous lamivudine therapy for over one year rebounded after withdrawal of drug, indicating that cccDNA was still present (Tyrrell, unpublished results). Quantitation of cccDNA from serial liver biopsies in ducks in which DHBV replication was inhibited using lamivudine and a ddG prodrug demonstrated a cccDNA pool half-life of approximately 50 days (1). The results of these studies indicate that elimination of HBV infection requires long-term antiviral therapy.

Unfortunately, extended lamivudine monotherapy results in the selection of lamivudine-resistant HBV variants. The incidence of lamivudine resistance was reported to be 16-32% among chronically infected individuals treated with lamivudine for one year (3). Longer-term studies have shown resistant rates as high as 58% and 49% after 104 weeks and 3 years, respectively, of lamivudine therapy (32). Lamivudine resistance has also been demonstrated in liver transplant patients (7, 34, 53) with the rates of resistance post-transplant as high as 60% at 11 months (36).

Mutations associated with lamivudine resistance occur most often in the conserved tyrosine-methionine-aspartic acid-aspartic acid (YMDD) motif of the nucleotide-binding site of the viral polymerase (2, 18, 34, 38). Single nucleotide changes at codon 204 of the reverse transcriptase domain of the polymerase result in the substitution of either valine or isoleucine for methionine (rtM204V or rtM204I). The valine substitution, and occasionally the isoleucine substitution, is accompanied by an additional upstream mutation at codon 180 where a methionine is substituted for a leucine (rtL180M) (2). The development of resistance to lamivudine in the human immunodeficiency virus (HIV) has been shown to involve similar mutations at the YMDD motif, both *in vitro* and *in vivo* (11, 46, 54). The appearance of lamivudine-resistant HBV suggests that lamivudine therapy must be combined with other antiviral drugs to delay the emergence of resistant mutants in patients on long-term therapy for chronic HBV infection. The ultimate goal of therapy is to suppress HBV replication long enough to allow decay of the cccDNA pool.

The stable HBV-producing human hepatoblastoma cell line 2.2.15(48), which carries HBV DNA integrated into the genome of HepG2 cells, has been used successfully to evaluate the effects of antiviral drugs on HBV replication (13, 17). PDHs from ducks congenitally infected with DHBV have also been used to screen drugs for antiviral activity against hepadnaviruses (10, 52). However, neither of these systems can be used to study the effect of antivirals on lamivudine-resistant HBV. Currently, analysis of lamivudine-resistant HBV involves tedious transient transfections or recombinant polymerase systems (2, 42). A tetracycline-inducible stable cell line expressing HBV containing the rtM204V mutation has been reported, however this mutation alone is not seen clinically and so is not ideal for screening antiviral compounds (28). Fu *et al* have also described stable cell lines expressing lamivudine-resistant HBV as an antiviral screening system (19). However, this study did not include the clinically relevant rtM204I mutation associated with lamivudine resistance. Recently, an *in vitro* system using recombinant baculoviruses to deliver the HBV genome into cells has been shown to be useful for testing antiviral compounds (15). Although this system is more efficient than transient transfections, it still requires the generation of recombinant baculoviruses and subsequent infection of cultured cells prior to the drug analysis. The purpose of this study was to produce stable cell lines expressing lamivudine-resistant HBV which could be used to easily screen drugs for antiviral activity against the lamivudine-resistant HBV.

2.2 Materials and Methods.

2.2.1 Plasmids and Mutagenesis.

An HBV genomic DNA construct was derived from pKS-HBV1 (47) which contains the HBV subtype adw. A 1.7mer HBV genome was cloned into pcDNAI/Amp (Invitrogen, Carlsbad, California) to generate the plasmid pCMV-HBVwt. An *XhoI/SalI* fragment, containing a neomycin resistance gene, from pMCIneoPolyA (Stratagene, LaJolla, California) was cloned into the *NheI* site of pCMV-HBVwt to generate pCMV-HBVwt-neo[2] (Figure 2.2). In this plasmid the CMV enhancer/promoter drives transcription of the HBV pregenomic RNA. Standard cloning techniques, as described in Sambrook, were used to generate these constructs (44).

Lamivudine-resistant mutations were introduced into the HBV genome using the Altered Sites *in vitro* Mutagenesis kit (Promega, Madison, Wisconsin). This system employs a phagemid, pAlter-1, which contains two genes for antibiotic resistance. The gene encoding tetracycline resistance is always functional and is used to select for the plasmid. The gene encoding ampicillin resistance has been inactivated. The mutagenesis reaction involves annealing both an oligonucleotide which restores amplicillin resistance and the mutagenic oligonucleotide to the single-stranded DNA template. Subsequent DNA synthesis and ligation links the two oligonucleotides. The DNA is then transformed into a strain of *E. coli* defective in mismatch repair and grown in the presence of ampicillin. A second round of transformation into *E. coli* DH5α results in segregation of wild-type and mutant plasmids.

A monomer HBV genome from *SphI*-digested pKSV-HBV1 was cloned into pAlter-1 (Promega) to generate pAlt-HBVwt[2]. To generate single-stranded DNA, pAlt-HBVwt was transformed into JM103 and plated onto LB-agar containing 12.5③g/ml tetracycline. A single colony was used to inoculate 5 mls of LB containing tetracyline and grown at 37°C overnight with shaking. The helper phage M13K07 was then added to the culture and allowed to absorb at 37°C for one hour without shaking. The culture was added to 100 ml of superbroth containing 12.5 μg/ml tetracyline and 50 μg/ml of kanamycin and incubated overnight at 37°C with vigorous shaking. Culture supernatant

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[2] These constructs were generated by Graham Tipples.

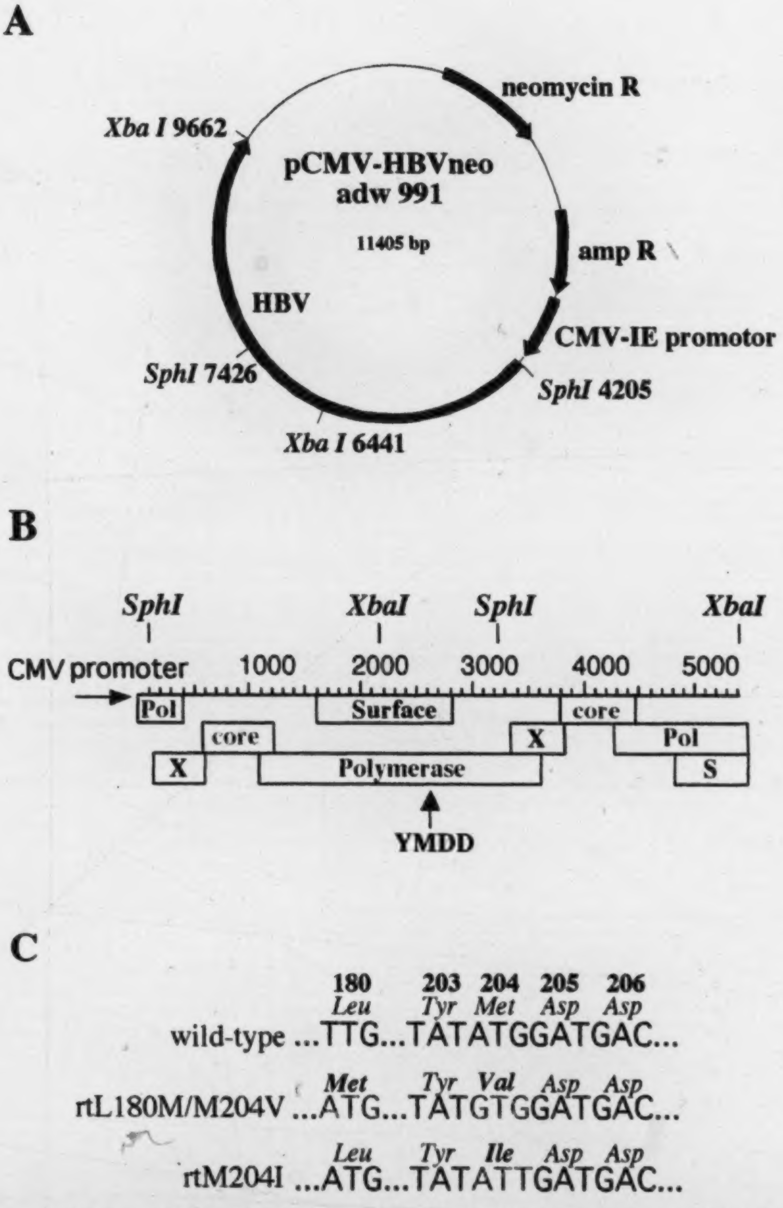
A
pCMV-HBVneo
adw 991
11405 bp
neomycin R
amp R
CMV-IE promotor
SphI 4205
Xba I 6441
SphI 7426
HBV
Xba I 9662
B
SphI
XbaI
SphI
XbaI
CMV promoter
1000
2000
3000
4000
5000
Pol
Surface
core
core
X
Pol
X
Polymerase
S
YMDD
C
180
203
204
205
206
Leu
Tyr
Met
Asp
Asp
wild-type ...TTG...TATATGGATGAC...
Met
Tyr
Val
Asp
Asp
rtL180M/M204V ...ATG...TATGTGGATGAC...
Leu
Tyr
Ile
Asp
Asp
rtM204I ...ATG...TATATTGATGAC...

Figure 2.2. Map of pCMV-HBV-neo construct used to generate stable cell lines rtM204I and rtL180M/M204V. A. Schematic diagram of the replication-competent HBV construct shows the cytomegalovirus immediate early promotor, HBV sequences, ampicillin-resistant marker and neomycin-resistant marker. **B.** Diagram depicting organization of open-reading frames in HBV sequences. **C.** Nucleotide sequence of wild-type and mutant viruses. Altered nucleotides are depicted in red. Numbers above depict aa from N-terminus of reverse transcriptase domain of polymerase protein.

supernatant was harvested by pelleting cells at 12,000 g for 10 minutes, transferring the supernatant to clean tubes and spinning for an additional 10 minutes. The phage was precipitated by adding 0.25 volumes of phage precipitation solution (3.75 M ammonium acetate, pH 7.5, 20% PEG 80000) to the cleared supernatant. The mixture was placed on ice for 30 minutes and then centriguged for 30 minutes at 12,000 g. The pellet was resuspended in 500 µl of TE buffer pH 8 and subjected to phenol:chloroform (1:1) extraction. This process was repeated until the interphase was clear (indicating the removal of all PEG) and was followed by a final extraction with chloroform. The DNA was precipitated by the addition of 1/10 volume 3 M sodium acetate and 2.5 volumes ethanol. The DNA was then pelleted, washed with 70% ethanol and resuspended in 50 µl of water. The integrity and quantity of the single stranded DNA was analysed using an ethidium-stained agarose gel.

The mutagenic and the ampicillin repair oligos were phosphorylated using T4 kinase and annealed simultaneously to the single stranded pAlt-HBVwt DNA. The mutagenic oligonucleotides used for the site-directed mutagenesis were:

L180M 5'-AGTCCGTTTCTC**ATG**GCTCAGTTTAC-3'
M204V 5'-CAGCTAT**GTG**GATGATGTGG-3'
M204I 5'-CAGCTAT**ATT**GATGATGTGG-3'

The annealing reaction contained 100 ng of single stranded DNA, 0.5 pmol of ampicillin repair oligo, 1 pmol mutagenic oligo and 1x annealing buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl). The mixture was heated to 70°C for five minutes, cooled to room temperature for 20 minutes and then placed on ice. T4 DNA polymerase, T4 DNA ligase and 1x synthesis buffer (10 mM Tris-HCl pH 7.5, 500 µM dNTPs, 1 mM ATP, 2 mM DTT) were added to the annealing reaction and incubated at 37°C for 90 minutes. The entire mixture was then transformed into BMH71-18 *mut S* and the cells grown in LB containing ampicillin overnight at 37°C with shaking. One-fifth of the plasmid DNA purified from the overnight culture was used to transform DH5α. The resulting ampicillin-resistant colonies were screened for the presence of the desired mutation by DNA sequencing. The mutagenesis generated the plasmids pAlt-HBVrtL180M, pAlt-HBV-rtM204V, and pAlt-HBV-rtM204I[3]. The corresponding mutations in the HBV polymerase are rtL180M, M204V, and M204I, respectively, based on the consensus nomenclature for HBV polymerase mutations (51). The mutations created in the overlapping surface antigen reading frame are S171S (no change), I195M, and W196L, respectively.

Cloning of fragments from pAlt-HBV-rtL180M, pAlt-HBV-rtM204V, and pAlt-HBV-rtM204I into pCMV-HBVwt-neo generated the plasmids pCMV-HBV-rtL180M/M204V-neo and pCMV-HBV-rtM204I-neo[3]. The nomenclature of the plasmids is such that the amino acid substitution and the position of the substitution is indicated. For example, pCMV-HBV-rtL180M/M204V contains a leucine to methionine substitution at amino acid 180 of the reverse transcriptase domain as well as a methionine to valine substitution at amino acid 204. Plasmids were linearized with *PvuI*, phenol/chloroform extracted and precipitated with 1/10 volume 3 M ammonium acetate and 2.5 volumes 95% ethanol prior to use in transfection experiments.

2.2.2 Cell culture, transfection and clone selection.

HepG2, a human hepatoma cell line (ATCC, catalogue number HB8065), cells were cultured a 37°C/ 5% CO_2 in minimal essential media (MEM) (ICN Biomedicals, Costa Masa, California) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Gaithersburg, Maryland), 2mM L-glutamine, 100 μg/ml streptomycin and 50 IU/ml penicillin G and buffered with sodium bicarbonate (complete media). The cell line 2.2.15 was cultured in complete media supplemented with 300-500 μg/ml of G418 (Life Technologies) as a selective agent.

HepG2 cells were seeded into 6-well plates or 60-mm culture dishes at approximately 60-70% confluence 12-24 hours prior to transfection. Linearised plasmid DNA was used to transfect the HepG2 cells using either the calcium phosphate method (44), or Lipofectin reagent (Life Technologies) according to the manufacturer's instructions. For transfection of HepG2 cells in 6-well plates using the calcium phosphate method, 5 μg of plasmid DNA was resuspended in a final volume of 250 μl water + 125 µl $CaCl_2$. An equal amount of 2x HEPES-buffer (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4-$2H_2O$, 12 mM dextrose, 50 mM HEPES pH 7.05) was then added drop-wise. After a five minute incubation at room temperature the DNA-calcium phosphate precipitate was added to the cells and incubated at 37°C overnight. Cell monolayers were trypsinised 24 hours after transfection and transferred into T25 flasks. For transfection of HepG2 cells in 60- mm dishes using Lipofectin, 10 µg plasmid DNA was resuspended in 200 µl serum-free media. Twelve microlitres of lipofectamine was resuspended in 200 µl of serum-free media. The DNA and lipofectamine mixtures were then combined and incubated at room temperature for 15-20 minutes. Serum-free media, 1.6 mls, was added and the entire mixture (2 mls) was added to the cells and incubated at 37°C overnight. Again, monolayers were trypsinised 24 hours after transfection and transferred to 100-mm dishes. Stable transformants were selected by growth in complete medium containing 500 µg/ml G418. Isolated clones were then cultured and expanded. Culture supernatant was subsequently assayed for production of viral antigens and extracellular viral DNA to select for HBV-producing clones. The calcium phosphate method of transfection generated more G418-resistant clones than the Lipofectin method.

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[3] These constructs were generated by Graham Tipples and Kathie Walters.

2.2.3 Characterisation of individual clones.

Cell culture supernatant was assayed for both HBV surface antigen (HBsAg) and e antigen (HBeAg) (Heprofile HBsAg and Heprofile HBeAg, ADI diagnostics, Willowdale, Ontario)[4]. HBV DNA in the cell culture supernatant was quantitated using a PCR-based antigen capture system as previously described (24).

2.2.4 Analysis of intracellular viral DNA.

Confluent monolayers of individual clones were rinsed with phosphate buffered saline (PBS). Cells were then lysed using 10 mM Tris-HCL pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.25% NP-40 and 8% sucrose. The lysates were transferred to microtubes and the nuclei pelleted by centrifugation at 14,000 rpm in an Eppendorf Centrifuge 5415 C microfuge for 4 minutes. The supernatants were transferred to clean microtubes and $MgCl_2$ concentration adjusted to 6 mM. DNase and RNase were added to 100 µg/ml and 10 µg/ml, respectively, and the mixture incubated at 37°C for 30 minutes. The samples were centrifuged as before and the lysate transferred to clean tubes. The virus was precipitated by adding 26% polyethylene glycol 8000, 1.4 M NaCl, 25 mM EDTA and incubating the sample at 4°C overnight followed by centrifugation as above. The pellets, containing virus and contaminating proteins, were resuspended in 50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM EDTA. Proteinase K and SDS were added to 800 µg/ml and 0.1%, respectively, and the mixture incubated at 42°C overnight. The samples were extracted with phenol/chloroform and the DNA precipitated using 1/10 volume 3 M sodium acetate/ 10 µg yeast tRNA/ 2.5 volumes 95% ethanol. Samples were separated on an 0.8% agarose gel in 0.5x TBE, depurinated for fifteen minutes in 0.25 M HCl, and transferred by capillary action onto Hybond N+ membranes (Amersham, Buckinghamshire, England) using 0.4 M NaOH. Membranes were pre-hybridized overnight in 5x SSPE, 2% SDS, 1x Denhardt's solution and 50 µg/ml herring sperm DNA at 65°C. HBV sequences were detected by hybridization with a nick-translated, ^{32}P-labelled 3.2 kb HBV probe. Membranes were washed twice with 2x SSPE 0.1% SDS and twice with 0.2x SSPE 0.1% SDS. Washes were 15 minutes each and were done with each solution first at room temperature and then at 65°C.

2.2.5 Integration of HBV DNA.

The integration of HBV genomic sequences into the host cell DNA was examined by Southern blot analysis of cellular genomic DNA. Genomic DNA was prepared by lysing cell monolayers with 10 mM Tris-HCl pH8, 1 mM EDTA, 0.1% Sarkosyl and digesting with 500 µg/ml Proteinase K at 42°C overnight. The sample was then deproteinated by extraction with an equal volume of phenol:chloroform (1:1) followed by a final extraction with chloroform alone. The DNA was precipitated using 0.2 M NaCl and 2 volumes of 95% ethanol. For Southern analysis, approximately 7 µg of genomic DNA was digested with *NsiI* and the resulting fragments separated and blotted as described above.

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[4] The assays for HBV sAg and eAg were done at the Provincial Laboratory, University of Alberta.

2.2.6 Antiviral Compounds.

The nucleoside analogues lamivudine, penciclovir, abacavir, and CDG were obtained from GlaxoWellcome, Research Triangle Park. The structures of all compounds used are shown in Figure 2.1. Stock solutions of drugs were stored at -80°C in DMSO. Lamivudine was used at final concentrations of 3.2, 16, 80, 400, and 2000 nM for wild-type virus (2.2.15 cells) and 3.2, 16, 80, 400, and 2000 µM for the mutant viruses (M552I and L528M/ M552V). Final concentrations of penciclovir used were 2.4, 12, 60, 300, and 1500 µM for both wild-type and mutant virus. Final concentrations of 1592U89 used were 0.32, 1.6, 8.0, 40, and 200 µM for the wild-type virus and 1.6, 8.0, 40, 200, and 1000 µM for the mutant viruses. Two different series of final CDG concentrations were used: 3.2, 16, 80, 400 and 2000µM for both wild-type and mutant viruses (results depicted in Table 2) or 0.128, 0.64, 3.2, 16, and 80 µM for both wild-type and mutant virus (results mentioned in Foot Note to Table 2).

2.2.7 Drug Assay.

Cells of the 2.2.15, rtM204I or rtL180M/M204V cell lines were seeded in 96-well plates at a concentration of 75,000 cells/well in 150 µl complete media + 300-500 µg G418 and allowed to grow to confluence. Four or seven days after seeding, medium was replaced with 150 µl complete media containing reduced serum (2%). Serum-reduced medium was used for the remainder of the drug study. Day 1: one week after seeding, medium was replaced with either medium alone or medium containing the desired concentration of antiviral compound. Day 3 and 5: cells were fed with fresh media alone or with media containing the antiviral compound. Day 7: medium was harvested from the cells and HBV DNA present in the culture supernatant quantitated as previously described[5] (24). Media samples were also analysed for the production of HBsAg and HBeAg.

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[5] Quantitation of extracellular HBV DNA was done by Shelly Allen at GlaxoSmithKline, RTP, North Carolina.

2.3 Results

2.3.1 Generation and characterisation of stable HBV-producing clones.

Transfection of HepG2 cells with pCMV-HBV-rtL180M/M204V-neo and pCMV-HBV-rtM204I-neo, depicted in Figure 2.2, resulted in the establishment of individual stable clones. Approximately 200 G418-resistant colonies were selected and cultured. Over time the majority of the clones died, leaving 3 clones for the pCMV-HBV-rtM204I-neo construct and 10 clones for the pCMV-HBV-rtL180M/M204V-neo construct. Potential HBV-producing clones were first assayed for the ability to produce the viral antigens, HBsAg and HBeAg, using an ELISA-based system. Of the three clones for the pCMV-HBV-rtM204I-neo, only one produced high levels of both antigens, while the remaining two produced low levels. Nine of the ten clones generated from the pCMV-HBV-rtL180M/M204V-neo construct produced high levels of antigens. Antigen-producing clones were then analysed for production of extracellular virus using an antigen-capture assay (24). The one rtM204I clone produced detectable levels of extracellular HBV DNA, while three of the rtL180M/M204V clones produced extracellular HBV DNA. The highest producer of these was used in the subsequent studies.

Table 2.1 shows the levels of viral antigen and extracellular (ECV) HBV DNA produced by the established cell lines. The control used in this study was the 2.2.15 cell line which stably expresses wild-type HBV. HBsAg and HBeAg were produced at comparable levels in all cell lines. When serum-reduced medium was added four days post-plating, 2.2.15 cells produced an average of 40.6 pg/ml of HBV DNA in the culture supernatants of untreated (no drug) wells 14 days post-plating. The level of HBV DNA present in the culture supernatant of the cell lines expressing the mutant viruses was consistently lower than that produced by the 2.2.15 cells. The rtM204I cell line produced an average of 3.5 pg/ml of viral DNA. The rtL180M/M204V cell line produced an average of 6.5 pg/ml of viral DNA. Delaying addition of serum-reduced media until seven days post-plating increased ECV DNA levels of the rtM204I and rtL180M/M204V viruses to 12.85 pg/ml and 61.25 pg/ml, respectively. Interestingly, a similar effect was not observed in production of wild-type virus from 2.2.15 cells. Although equal numbers

TABLE 2.1. Production of HBsAg, HBeAg and extracellular viral DNA.

Cell Line	HBsAg	HBeAg	HBV DNA
2.2.15 (wt)	82.0 +/- 28.6	116.3 +/- 51.7	40.6 +/- 39
rtM204I	69.6 +/- 26.4	79.2 +/- 30.0	3.5 +/- 0.5
rtL180M/M204V	40.3 +/- 15.5	126.9 +/- 34.7	6.5 +/- 2.5

Viral antigens are expressed as ng/ml and viral DNA is expressed as pg/ml. Results are expressed as mean and standard deviation based on a minumum of two independent experiments done in triplicate. Values represent accumulation over 48 hours in culture media from confluent cell monolayers.

of cells were seeded for each cell line, it was noted that the rtL180M/M204V and rtM204I cell lines grew at slightly slower rates than the 2.2.15 cells. Maximum virus production is achieved when cells are not dividing (personal observation). Delaying the addition of serum-free media may give the slower-growing cells time to achieve complete confluency and hence become stationary. Since 2.2.15 cells are likely able to achieve stationary phase, and hence maximum virus production, by day 4 they are not affected by the delay in addition of serum-free media.

Analysis of the intracellular viral replicative intermediates, seen in Figure 2.3, shows the relaxed circular (rc), double-stranded linear (l) and single-stranded (ss) forms of the viral DNA. Both mutant viruses produce less viral DNA of all forms than the wild-type virus, although the difference is not as great as seen with the ECV levels.

Stable integration of the HBV genome in each of the cell lines was confirmed by isolating genomic DNA, digesting it with *NsiI*, and analyzing the resulting fragments by Southern hybridisation. There is a single recognition site for *NsiI* present within the HBV sequence but none in the vector sequence. Hybridisation with a probe consisting of only HBV sequences should result in two bands for every copy of integrated HBV sequences, assuming the copies are not in tandem. Figure 2.4a shows the Southern analysis of genomic DNA of the stable cell lines. Both the rtM204I and rtL180M/M204V cell lines, shown in lanes two and three respectively, showed more than two bands of HBV-specific DNA sequences. They both also contain intense bands of 11 and 9.5 kbp, respectively. This is approximately equivalent to the size of the CMV-HBV-neo constructs, which likely indicates that both cell lines contain multiple integrated copies of the plasmid arranged in tandem. Both cell lines contain a band of approximately 3.9 kbp (the band is evident in the rtM204I cell line upon a longer exposure, data not shown). This band was also seen in other stable HBV cell lines that were not used in this study (data not shown). It likely represents the relaxed-circular form of the viral DNA, which also runs at 3.9 kbp, and so was not considered when examining the integration of HBV sequences. As expected, HepG2 cellular DNA, shown in lane one, contains no HBV-specific sequences. The experiment has been repeated with other restriction enzymes that cut either in vector (*Hind III*) or HBV sequences (*Xba I*) (Figure 2.4b). The results

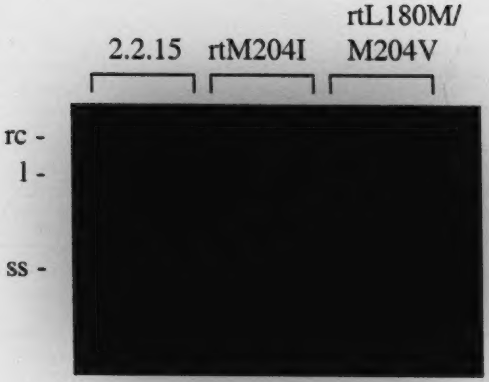

Figure 2.3. Production of intracellular viral replicative intermediates in 2.2.15, rtM204I and rtL180M/M204V cell lines. Intracellular viral DNA was harvested from confluent monolayers of 2.2.15, M204I and L180M/M204V cell lines and analysed by Southern blot. The probe consisted of a ^{32}P-labelled HBV monomer sequence. Results depict duplicates of the same experiment. The relaxed circular (rc), double-stranded linear (l), and single-stranded (ss) forms of the viral DNA are indicated.

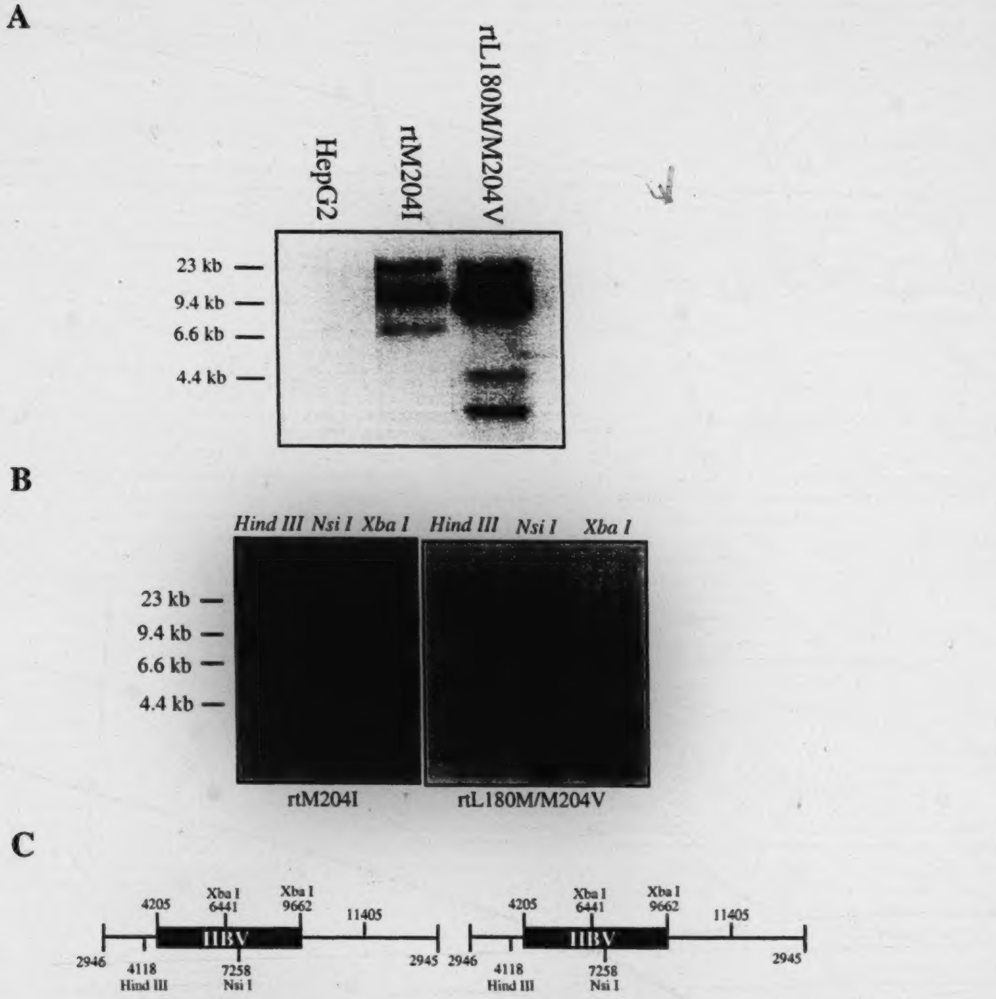
A
HepG2
rtM204I
rtL180M/M204V
23 kb
9.4 kb
6.6 kb
4.4 kb
B
Hind III
Nsi I
Xba I
Hind III
Nsi I
Xba I
23 kb
9.4 kb
6.6 kb
4.4 kb
rtM204I
rtL180M/M204V
C
4205
Xba I 6441
Xba I 9662
11405
HBV
2946
4118 Hind III
7258 Nsi I
2945
4205
Xba I 6441
Xba I 9662
11405
HBV
2946
4118 Hind III
7258 Nsi I
2945

Figure 2.4. Integration of HBV sequences into genomic DNA of HepG2, rtM204I and rt L180M/M204V cell lines. A. Genomic DNA from confluent monolayers was isolated, digested with *NsiI* and analysed by Southern blot for the presence of HBV sequences. The probe consisted of a ^{32}P-labelled HBV monomer sequence. **B.** Genomic DNA from confluent monolayers of either rtL204M or rtL180M/M204V was isolated, digested with *Hind III*, *Nsi I*, or *Xba I* and analysed by Southern blot as described above. **C.** Schematic of head-to-tail integration of CMV-HBVneo constructs linearised with *Pvu I*. Numbers represent nucleotide posititions of restriction enzymes and HBV sequences.

indicate the presence of both head-to-head and head-to-tail repeats of the HBV construct in the cellular genome of the stable cell lines. A schematic of the map of a head-to-tail repeat is shown in Figure 2.4C. When digested with *Nsi I* and probed with HBV sequences only, the pattern on a Southern blot should include a band of approximately 11 kb as well as two additional bands of undetermined size.

2.3.2 Analysis of antiviral activity.

The cell lines producing the rtM204I and rtL180M/M204V viruses were used to screen for antiviral activity of lamivudine and several other antiviral agents to determine if this system would be appropriate for antiviral drug screening. Lamivudine was used to confirm that the mutations introduced conferred lamivudine resistance. Purine-based analogues, particularly guanosine analogues, have been shown to be more effective anti-HBV agents, possibly because they have the potential to target both the protein priming and DNA synthesis steps (23). As protein priming and reverse transcription are separate biochemical reactions involving different regions of the polymerase protein (55), it is possible that the activity of purine analogues may be less affected by mutations in the YMDD motif. The purine analogues penciclovir, abacavir and CDG were used to determine if the lamivudine-resistant mutations also conferred resistance to purine analogues. Each cell line was treated with the antiviral compounds for one week. Culture supernatant was then removed and the HBV ECV DNA was quantitated. The 2.2.15 cell line was used as the wild-type HBV control.

Table 2.2 shows the effect of the various drugs on wild-type and mutant virus production. The IC_{50}s were estimated by plotting the viral DNA concentration (pg/ml) versus the log of drug concentration. The curve was fitted using the Hill equation $y=V_{max}(1-(x^n/(k^n + x^n)))$ using nonlinear regression to estimate the IC_{50}. A representative of the graphs showing the IC_{50}s obtained from two experiments using the rtL180M/M204V cell line are shown in Figure 2.5. An IC_{50} of 7.2 nM for lamivudine was obtained for the wild-type virus. IC_{50} values of 3.3 μM and 23 μM for lamivudine were obtained for the rtM204I and rtL180M/M204V viruses, respectively. These values are comparable to those obtained in previous studies and confirmed that viruses containing these mutations showed a marked insensitivity to lamivudine (2, 27, 42). An IC_{50} value of 45.9 μM was

TABLE 2. Effect of nucleoside analogues on wild-type and lamivudine-resistant HBV.

Cell Line	IC_{50} Lamivudine	IC_{50} Penciclovir	IC_{50} Abacavir	IC_{50} CDG[a]
2.2.15(wt)	0.0072 +/- 0.0029	45.9 +/-32	3.4 +/- 1.4	0.0009 +/- 0.0003
rtM204I	3.3 +/- 2.2	394.8 +/-213	3.6 +/- 0.7	< 0.0032
rtL180M/M204V	23 +/- 6.0	236 +/- 93	4.1 +/- 2.2	< 0.0032

IC_{50} is the concentration of drug which inhibits HBV DNA production by 50% compared to drug-free cultures. Concentrations are given in uM.

Results are given as means and standard deviation calculated from between 2 and 6 independent experiments each done in triplicate.

[a] In separate experiments done under slightly different conditions than those represented in the table (See Materials and Methods), IC_{50} values of 0.002, 0.003, and 0.002 were obtained for the wild-type, rtM204I, and rtL180M/M204V viruses, respectively, for CDG.

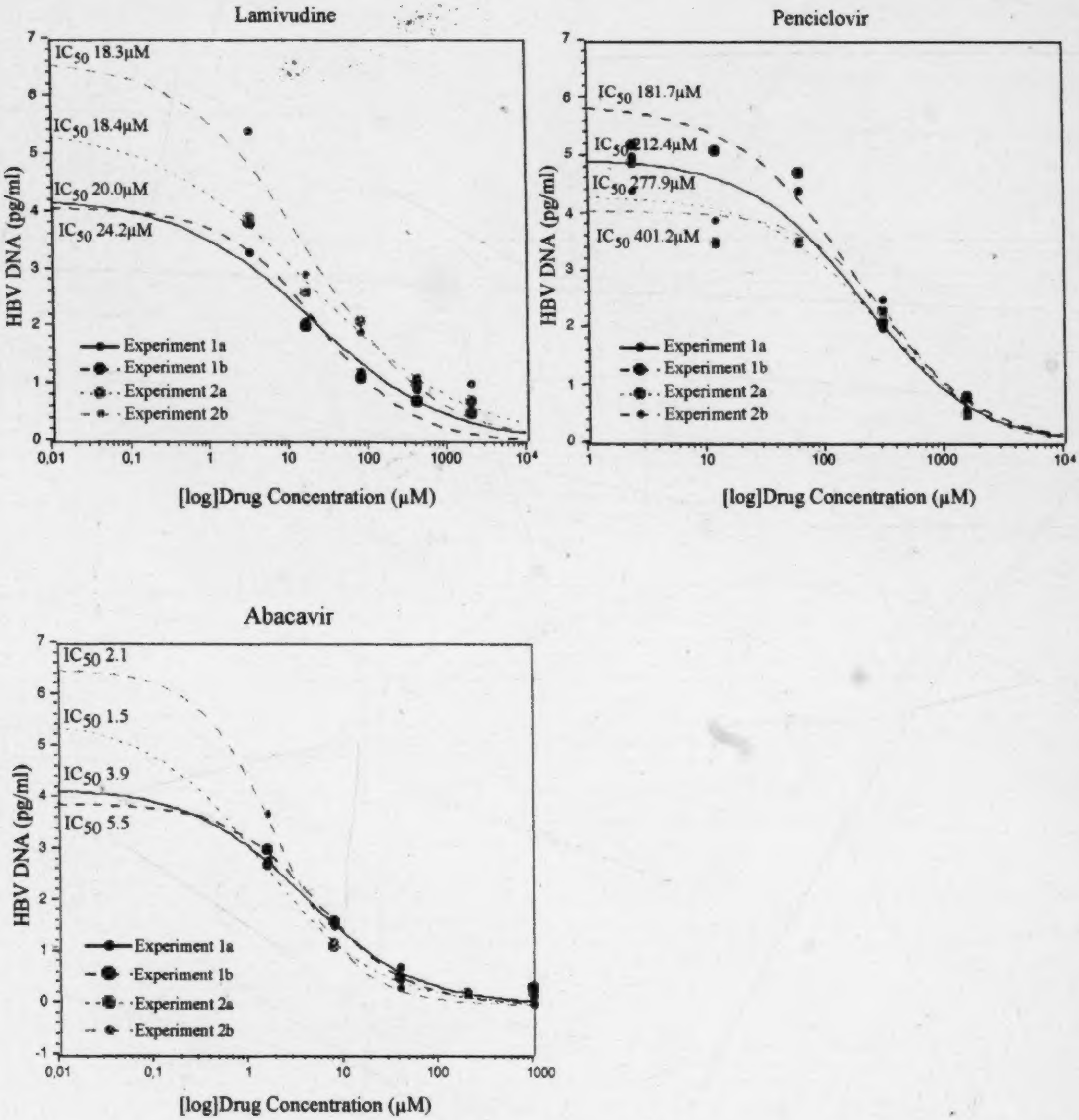

Figure 2.5: IC_{50} graphs for lamivudine, penciclovir and abacavir using the rtL180M/m204V cell line. The HBV DNA concentration (pg/ml) versus log of drug concentration were plotted using Kalidagraph. The curve was fitted using the Hill equation $y=V_{max}(1-((x^n)/(k^n+x^n)))$ using nonlinear regression to estimate the IC_{50}. Each graph represents the data from two independent experiments done in duplicate.

achieved for penciclovir with the wild-type virus and 394.8 μM and 236.7 μM for the rtM204I and rtL180M/M204V viruses, respectively. The high standard deviation values obtained may be due to the fact that the activity of penciclovir is affected by the growth rate of the cells (Shelley Allen, personal communication), which likely differs slightly between experiments. The IC_{50}s obtained for abacavir were 3.4 μM for the wild-type virus and 3.6 μM and 4.1 μM for the rtM204I and rtL180M/M204V viruses, respectively. Analysis of CDG resulted in an IC_{50} of 0.9 nM for the wild-type virus, and less than 3.2 nM for both the rtM204I and rtL180M/M204V viruses. To obtain more accurate IC_{50} values, lower concentrations of CDG were used in a second experiment (Results are shown in the Footnote to Table 2). The IC_{50} values were 2 nM, 2.98 nM, and 2.4 nM for the wild-type, rtM204I, and rtL180M/M204V viruses, respectively. IC_{50} values obtained for all drugs were well below previously published toxicity levels for the type of cells used in this study (14, 26, 28).

2.4 Discussion

Transfection of HepG2 cells with pCMV-HBV-rtM204I-neo and pCMV-HBV-rtL180M/M204V-neo gave rise, respectively, to the stable cell lines rtM204I and rtL180M/M204V. These cell lines produced both intracellular and extracellular virus, although generally at lower concentrations than the control 2.2.15 cell line.

The quantitation of ECV in this study involved binding of virus to anti-HBsAg-coated plates (24). However, the lower level of virus seen with the mutant viruses was unlikely due to differences in binding properties of the mutant viruses as Melagari *et al* (38) showed that the mutations introduced into the overlapping HBsAg reading frame did not affect the binding properties of HBsAg. This conclusion is further supported by the lower level of viral replicative intermediates seen in Southern analysis of intracellular virus.

The impaired polymerase activity associated with the mutant viruses may, at least partially, account for the lower virus production in cell lines producing the mutant viruses HBV containing the rtM204I or rtM204V mutations alone replicate at a significantly lower rate than the wild-type virus (27, 33, 38, 43). Virus containing the double rtL180M/M204I or rtL180M/M204V mutations replicate at a higher rate than virus containing the single mutation alone but not as efficiently as wild-type virus (38) This may also explain the consistently higher production of virus from the rtL180M/M204V cell line relative to the rtM204I cell line.

Another contributing factor for the differences in virus levels produced by the cell lines may be related to the nature of the integration of the HBV sequences. Both the number of integrated copies of the HBV genome and the site of integration may influence virus production. Southern analysis showed that each clone likely has multiple tandem copies of the HBV sequences integrated in the host DNA. It also showed that for each clone, the integration positions were unique. Because different regions of the chromosome can have varying influences on the transcription activity of the integrated HBV genome, the integration site may affect the level of virus production. Therefore, individual clones cannot be directly compared for levels of virus production, nor can differences in their virus production be considered significant. With respect to the drug analysis, the levels of virus produced from each cell line are internally controlled and have no effect on the results because the IC_{50}s are calculated based on virus production from untreated cells for the same cell line.

A system of classifying the degree of change in susceptibility of a virus to an antiviral agent has previously been described (25) and was used in the present study. A three-fold decrease in sensitivity is classified to be no change, a decrease between three-fold and 10-fold is classified as reduced sensitivity, and a decrease of 10-fold or greater is classified as resistant (25).

Virus containing the mutations rtM204I and rtL180M/M204V exhibited resistance to lamivudine, consistent with previous observations (2, 18, 28, 42). A 450-fold increase in IC_{50}, as compared to the wild-type virus, was seen in virus containing the rtM204I mutation. A 3000-fold increase in IC_{50} was seen with virus containing the rtL180M/M204V mutations.

Ladner *et al* used a construct containing a cDNA copy of the pregenomic RNA of an HBV genome carrying the single rtM204V mutation to produce a stable tetracycline-inducible cell line (28). The shifts in IC_{50} for lamivudine seen in Ladner *et al* were smaller than those seen in the present study. It has been shown that lamivudine resistance conferred by the single rtM204V mutation is increased when combined with the upstream rtL180M mutation, from a 186-fold increase in IC_{50} to a >10,000-fold increase (2). Furthermore, the rtM204V mutation alone is rarely seen in clinical isolates of lamivudine-resistant HBV (2). Screening for antivirals still active against lamivudine-resistant HBV should therefore be done using the more clinically relevant rtM204I or rtL180M/M204V mutations.

The presence of the mutations also caused a decreased sensitivity to penciclovir. In general, the IC_{50} obtained for penciclovir in this study were higher than other reported IC_{50}s for penciclovir. The reason for this is not clear but may be related to the method of culturing the cells in the drug assay. A nine-fold increase in IC_{50} was seen with virus containing the rtM204I mutation. A five-fold increase in IC_{50} was seen with virus containing the rtL180M/M204V mutations. This is consistent with a previous report in which rtL180M/M204V was shown to have reduced sensitivity to penciclovir (19). The rtL180M mutation has been associated with penciclovir resistance (38) and the lack of significant difference in the IC_{50}s of the rtM204I and rtL180M/M204V viruses was surprising. The rtL180M/M204V virus might have been expected to exhibit a greater decrease in sensitivity to penciclovir than the rtM204I virus. Similar results were obtained in the study by Delaney *et al* where the single rtM204I mutation also exhibited a higher resistance to penciclovir compared to either the rtL180M or rt180M/M204V mutations (15). This observation suggests that the rtL180M mutation alone may be insufficient to cause penciclovir resistance. Indeed, the rtL180M mutation is just one of several mutations associated with penciclovir resistance (58). Despite the fact that the mutant viruses in the present study showed only a decreased sensitivity to penciclovir *in vitro*, patients with lamivudine-resistant virus show poor response to penciclovir treatment (22, 50). In addition, penciclovir is unlikely to be a good choice for combination therapy with lamivudine since resistance to each of the drugs arises from the common mutation rtL180M.

There was no significant shift in the IC_{50} values of either abacavir or CDG seen with either the rtM204I or rtL180M/M204V mutations as compared to the wild-type virus. HBV carrying the rtM204V mutation alone has also previously been shown to remain sensitive to CDG (28). This indicates that HBV resistant to lamivudine would likely be sensitive to treatment with either drug. Both drugs are therefore good candidates to be used in combination therapy with lamivudine. Abacavir and CDG are purine-based derivatives. Purine-based analogues have been shown to be more effective inhibitors of hepadnaviral replication than pyrimidine-based analogues (31, 52). Of the purine analogues, guanosine-based analogues may be the more effective anti-HBV agents as they have the potential to target two separate processes in HBV replication. In addition to acting as a DNA chain terminator, these drugs can also target the priming step of reverse transcription as the first nucleotide covalently bound to the primer protein is a guanosine residue (62). This may partially explain why changes in the YMDD motiff do not affect the anti-HBV activity of Abacavir or CDG. However, HIV resistance to abacavir has been observed and involves the equivalent M184V mutation that is responsible for resistance to lamivudine. It is not clear why the corresponding mutation in HBV remains sensitive to abacavir in this system. It is possible that additional mutations not yet recognised in HIV are required for the resistance. Alternatively, it is possible that additional mutations not found in HIV are required for similar resistance to abacavir by HBV. An example of this is the upstream rtL180M mutation accompanying the rtM204V mutation associated with HBV but not HIV lamivudine resistance.

A recent study using recombinant human HBV polymerase demonstrated that the mutations associated with both penciclovir and lamivudine-resistance do not confer resistance to the purine-based analogue adefovir (57). Lamivudine-resistant HBV has also been shown to remain sensitive to lobucavir (42). Different resistance profiles between the rtL180M/M204V and rtM204V viruses have been noted for L-FMAU. Ying *et al* have shown that HBV with the rtM204V mutation remains sensitive to L-FMAU while a separate study has shown that the rtL180M/M204V virus is cross-resistant (20, 59). This discrepancy emphasizes the importance of using clinically relevant mutations when screening antivirals.

The production of stable cell lines that express lamivudine-resistant HBV eliminates the need for tedious transfections before each drug screening experiment. Screening for antiviral activity against lamivudine-resistant virues can now be done with relative ease. There are, however, a few draw-backs to this system. Long-term drug analysis to assess the durability of the compound's antiviral effect is difficult due to the fact that maximal virus production is obtained from stationary cells. This requires that the cells be cultured in a confluent state with reduced serum which limits the length of the experiment to approximately 14 days. However, this drug assay system was designed primarily for use of preliminary screening. Long-term studies to assess durability of antiviral effects are best done in the woodchuck or duck animal models. Analysis of drug sensitivity is also limited to the genotype used to generate the stable cell lines. As well, because virus production is driven from an integrated template, it is highly unlikely that this system can be used to select for HBV variants resistant to antiviral compounds. To date, there have been no reports of lamivudine-resistant HBV being selected *in vitro*. As well, resistant HBV variants take months to emerge *in vivo* and while drug-resistance does emerge after long-term treatment in the woodchuck model *in vivo* (58, 60, 61), it has never been demonstrated in the duck model. The emergence of drug-resistanct HBV variants appears to be a relatively uncommon event requiring long-term therapy.

The results in this study showed that although viruses containing the rtM204I or rtL180M/M204V mutations are resistant to lamivudine, they are still sensitive to abacavir and CDG. These cell lines have also been used by Mehta *et al* to study a new class of anti-hepadnaviral agents called alcovirs (37). These compounds include N-nonyl-deoxy-galactonojirimycin (N-nonly-DGJ), a galactose-based imino sugar with a nine carbon alkyl side chain. The exact mechanism of the antiviral activity of these compounds is not known. However, the fact that the activity is not affected by the YMDD mutants suggests the mechanism is disctinct from that of nucleoside analogues such as lamivudine and penciclovir. They are therefore promising alternatives to other nucleoside analogues for treatment of lamivudine-resistant variants. With the recent licensing of lamivudine for treatment of HBV infection, widespread use of the drug has resulted in significant lamivudine-resistance in HBV-infected individuals. It is imperative that additional antivirals active against the YMDD mutants be identified, so that combination therapy can be used to prevent the emergence of lamivudine-resistant HBV isolates seen in patients on monotherapy.

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Chapter 3

Superinfection Exclusion in Duck Hepatitis B Virus Infection[1].

3.1 Introduction.

As mentioned in the previous chapter, lamivudine-resistant HBV variants occur as a result of prolonged monotherapy (1, 12, 19, 29). The nature of lamivudine resistance in HBV infection is unusual in several ways. Firstly, although the same mutation is responsible for both HIV and HBV lamivudine resistance (1, 30), the length of treatment before emergence of resistant HBV variants is substantially long compared to emergence of HIV resistance (months versus weeks, respectively). Secondly, it has been shown that the average length of lamivudine monotherapy before resistant HBV variants emerge is longer in non-transplant patients compared to transplant patients, 562 days versus 371 days, respectively (8). Lastly, the rates of resistance appear to be higher in transplant patients compared with non-transplant patients (6, 12, 23). These observations suggest that the lamivudine-resistant HBV variant establishes an infection in an uninfected liver more readily than in an HBV-infected liver. One possible explanation is that the immuno-suppressive therapy in transplant patients is enabling resistant virus to establish an infection more readily. However, chronic HBV infection is the result of an inefficient immune response against HBV. It is unlikely, therefore, that the immunosuppressive therapy would make much difference with regard to immune-mediated control of HBV replication in these patients. Another possible explanation is that once a cell is infected with HBV, it is no longer susceptible to further infection by the same virus. This phenomenon is known as superinfection exclusion and is seen with a number of viruses including HIV (13), vesicular stomatitis virus (34), vaccinia virus (4), and alphavirus (11). Recent studies using the DHBV animal model have also suggested that superinfection exclusion occur in hepadnavirus infections. Studies of viral dynamics in ducks have shown that enrichment of wild-type DHBV over replication-defective mutants is rapid during the initial spread of infection. Thereafter, the enrichment rate is slower and appears to be dependent on the generation of new uninfected hepatocytes (35, 36). As well, emergence of wild-type DHBV in competition studies with a cytopathic variant appears to be dependent on cell death caused by the mutant virus, as wild-type virus does not emerge in competition studies with a non-cytopathic variant (14). The DHBV animal model was used to investigate the possibility of superinfection exclusion in hepadnaviral infection.

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[1] A version of Chapters 3 and 4 have been submitted to Journal of Virology, June, 2003.

3.2 Materials and Methods.

3.2.1 Animals and virus stocks. Lamivudine was obtained from GlaxoSmithKline, Research Triangle Park, North Carolina. Newborn Pekin ducklings, either congenitally-infected with DHBV-16 or uninfected, were obtained from the University of Alberta, Edmonton, Alberta. All animals were screened for the presence of DHBV infection by dot-blot prior to use in these studies. Serum from strongly viremic congenitally-infected ducks was used as source for wild-type DHBV. Serum containing DHBV-*ClaI* or DHBV-M512V (7) was passaged several times in ducks to obtain high-titre serum. DHBV-*ClaI* contains a point mutation at nucleotide 1858, based on the Mandart numbering system of the DHBV genome (20), which introduces a *ClaI* restriction site without altering the amino acid sequence of the polymerase reading frame. When passaging DHBV-M512V, animals were maintained on lamivudine therapy at 40mg/kg intra-muscular (i.m.) twice daily to prevent reversion to wild-type virus. Viral titres were quanititated by dot-blot with plasmid standards and expressed as viral genome equivalents (VGE). All animals were infected by intra-muscular (i.m.) injections. Serum samples were taken weekly to monitor infection.

3.2.2 Extraction of extracellular viral (ECV) DNA from serum. Twenty µl of serum was incubated in 80 µl of 50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM EDTA, 0.1% SDS and 800 µg/ml Proteinase K at 42°C for a minimum 4 hours. The sample was then extracted with an equal volume of phenol/chloroform. DNA was precipitated by adding 1/10 volume 3M sodium acetate, 10 µg yeast tRNA and 2 ½ volumes 95% ethanol. The DNA was resuspended in 10 µl of water and 5 µl was used in a subsequent polymerase chain reaction (PCR).

3.2.3 Preparation and infection of primary duck hepatocytes. Primary duck hepatocytes (PDH) from 14-21 day -old ducklings were prepared using collagenase as previously described (27). Cells were plated at 750,000 cells per well in six-well plates and cultured at 37°C in L15 media supplemented with 1.2 µg/ml insulin, 1.7 µg/ml glucose, 11 µM hydrocortisone hemisuccinate, 15 mM Hepes, 5% FBS, 50 IU/ml penicillin, 10 µg/ml streptomycin and 25 µg/ml nystatin. One day post-plating the media was replaced with serum-free media and cultured for an additional two days. At three days post-plating, cells were infected with DHBV-positive serum at an MOI of 100-200 in media containing 1.5% dimethylsulfoxide. Infections were carried out overnight at 37°C and the media was then replaced with fresh serum-free media every second day.

3.2.4 Isolation of intracellular viral DNA from primary duck hepatocytes. Monolayers were first rinsed with phosphate buffered saline (PBS) and then lysed using 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.25% NP-40 and 8% sucrose. The lysates were transferred to microtubes and the nuclei pelleted by centrifugation at 14,000 rpm in an Eppendorf Centrifuge 5415 C microfuge for 4 minutes. The supernatants were transferred to clean microtubes and $MgCl_2$ concentration adjusted to 6 mM. DNase and RNase were added to 100 µg/ml and 10 µg/ml, respectively, and the mixture incubated at 37°C for 30 minutes. The samples were centrifuged as before and the lysate transferred to clean tubes. The virus was precipitated at 4°C overnight using 26% polyethylene glycol 6000, 1.4 M NaCl, 25 mM EDTA followed by centrifugation as above. The pellets, containing virus and contaminating proteins, were resuspended in 50 mM Tris-HCl pH 8, 150 mM NaCl, 10 mM EDTA. Proteinase K and SDS were added to 800µg/ml and 0.1%, respectively, and the mixture incubated at 42°C overnight. The samples were then extracted with phenol/chloroform. The DNA was precipitated as previously described and resuspended in 20 µl water. Five to 10 µl were used in subsequent PCR reactions or Southern blots (described in Chapter 2).

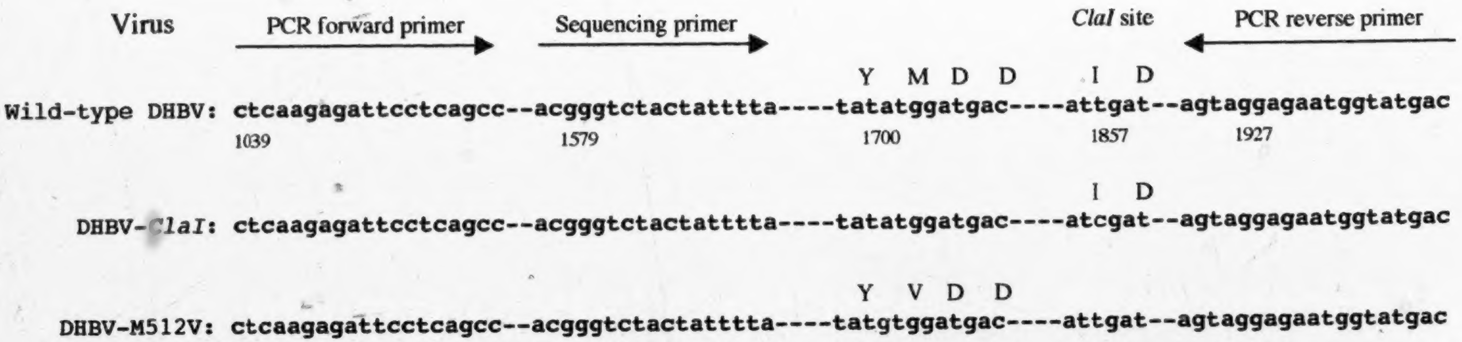
3.2.5 Analysis of viral DNA. The extracted viral DNA was amplified by PCR using the following primers:

Forward: 5'-ctcaagagattcctcagcc-3'(DHBV nucleotides1039-1058)
Reverse: 5'-gtcataccattctcctact-3' (DHBV nucleotides 1945-1926)

Each 50 µl PCR reaction contained 1x Taq buffer (Gibco BRL), 2.5 mM $MgCl_2$, 200 µM dNTPs, 12.5 pmol of each primer and 1 unit of Taq polymerase (Gibco BRL). Cycles for the PCR were as follows: 95°C for 4 minutes, then 30 cycles of [95°C for 30s, 50°C for 30s, 72°C for 1 minute] followed by 72°C for 7 minutes.

To distinguish between wild-type DHBV and DHBV-*ClaI*, 10 µl of PCR product was digested with the restriction enzyme *ClaI* at 37°C for at least 1 hour. The digestion products were then separated on a 1.3% agarose gel and visualized by ethidium bromide. To distinguish between wild-type DHBV and DHBV-M512V, the PCR products were sequenced. Partial nucleotide sequences of all DHBV variants depicting PCR and sequencing primer locations is shown in Figure 3.1.

3.2.6 Cell sorting and single cell PCR. Cells were washed twice with PBS, treated with a glycine buffer (50 mM glycine, 150 mM NaCl, pH 2.2) for 1.5 minutes to remove bound virus (Ref) and then washed twice more with PBS. They were then trypsinised, pelleted by centrifugation and washed twice more with PBS. The cells were counted and checked for viability using trypan blue exclusion. Single-cell PCR was performed using a modified version of a previously described protocol (31). The cells were sorted into 0.2 ml PCR tubes containing 10 µl of lysis solution (200 mM KOH, 50 mM DTT). The cells were heated at 65°C for 10 minutes, cooled briefly and neutralised with 10 µl of 400 mM Tris, 0.2 N HCl pH. They were then heated at 93°C for 15 minutes, cooled briefly and a PCR mixture was added to a final volume of 100 µl. The PCR conditions were as described below with the exception that 40 cycles were used for the single-cell PCR. PCR products were digested with *ClaI* and analyzed by gel electrophoresis or by Southern blot.



```
                                                                  Y  M  D  D    I  D
Wild-type DHBV: ctcaagagattcctcagcc--acgggtctactatttta----tatatggatgac----attgat--agtaggagaatggtatgac
                1039                 1579                 1700               1857    1927

                                                                                I  D
    DHBV-ClaI: ctcaagagattcctcagcc--acgggtctactatttta----tatatggatgac----atcgat--agtaggagaatggtatgac

                                                                  Y  V  D  D
   DHBV-M512V: ctcaagagattcctcagcc--acgggtctactatttta----tatgtggatgac----attgat--agtaggagaatggtatgac
```

Figure 3. 1: Partial nucleotide sequences of wild-type DHBV, DHBV-*ClaI* and DHBV-M512V. Nucleotide variations from wild-type virus are depicted in red. DHBV-*ClaI* contains a unique *ClaI* restriction site introduced by a single nucleotide change. DHBV-M512V contains a single nucleotide change that results in a substitution of a valine for a methionine at the YMDD region of the polymerase gene, conferring resistance to lamivudine (Fischer *et al*, 1996). The position and orientation for PCR and sequencing primers are indicated.

3.3 Results.

3.3.1 DHBV-*ClaI* establishes an infection in uninfected but not congenitally-infected animals. The ability of ducklings congenitally infected with DHBV to be superinfected with another DHBV was examined to determine if superinfection exclusion occurs in hepadnavirus infections. A genetically tagged virus, DHBV-*ClaI*, was used in these studies as it can be distinguished from the endogenous DHBV present in the animals. DHBV-*ClaI* contains a single nucleotide change which introduces a *ClaI* restriction site into the DHBV genome without altering the amino acid sequence of the polymerase ORF. To determine if an infection with DHBV-*ClaI* could be established in an animal already infected with the DHBV, high-titre serum containing DHBV-*ClaI* was used to inoculate congenitally-infected newborn ducklings. Five one-day old congenitally-infected ducklings were each injected with serum containing approximately 2 x 10^{10} VGE of DHBV-*ClaI*. Five uninfected animals of the same age were similarly infected as controls. Serum samples were taken weekly to monitor infection. Viral DNA was extracted from serum and amplified by PCR. The full-length PCR product is 906 bp. *ClaI* digestion of the PCR product from the DHBV-*ClaI* virus reduces the size of the product to 820 bp. The DHBV PCR product, which lacks the *ClaI* restriction site, remains at 906 bp. The two products can then be separated by gel electrophoresis. To determine the sensitivity of this assay, different ratios of two plasmids, pCMV-DHBV and pCMV-DHBV-*ClaI* were mixed, amplified by PCR and digested with *ClaI*. The amount of total DHBV DNA in each reaction was equivalent to 2 x 10^7 VGE. This amount represents the amount of viral DNA present in 20μl of serum processed in animal experiments, based on a viremia level of 10^9 VGE/ml. The results, shown in Figure 3.2, show that the PCR assay is able to detect 2% DHBV-*ClaI* in a background of DHBV, which is comparable to the sensitivity of assays used in other superinfection studies (33). Mixing different ratios of duck serum containing known levels of DHBV and DHBV-*ClaI* , extracting the viral DNA and analysing by PCR and *ClaI* digest yielded the same result (data not shown).

Figure 3.3a shows the results of analysis of serum viral DNA taken 14 days post-infection. The serum of the congenitally-infected animals (lanes one to five) showed no evidence of DHBV-*ClaI* superinfection since no 820bp product was detected. However,

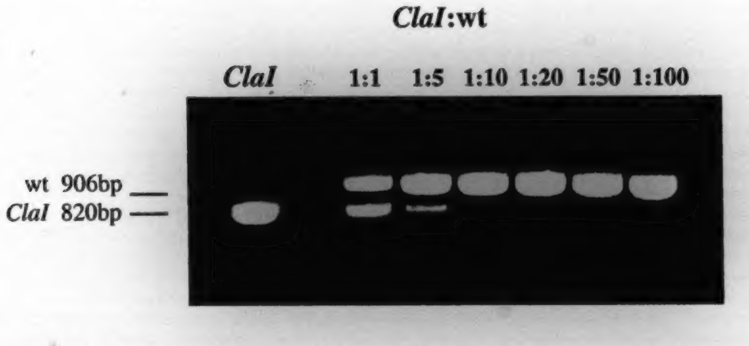

ClaI:wt
ClaI
1:1
1:5
1:10
1:20
1:50
1:100
wt 906bp
ClaI 820bp

Figure 3.2: Sensitivity of the PCR assay used to distinguish DHBV from DHBV-*ClaI*. Various ratios of the plasmids pCMV-DHBV and pCMV-DHBV-*ClaI* were mixed, amplified by PCR and digested with the restriction enzyme *ClaI*. The products were then separated using a 1.2% agarose gel and the DNA visualised by Ethidium bromide staining. *ClaI* lane represents pCMV-DHBV-*ClaI* control for complete digestion.

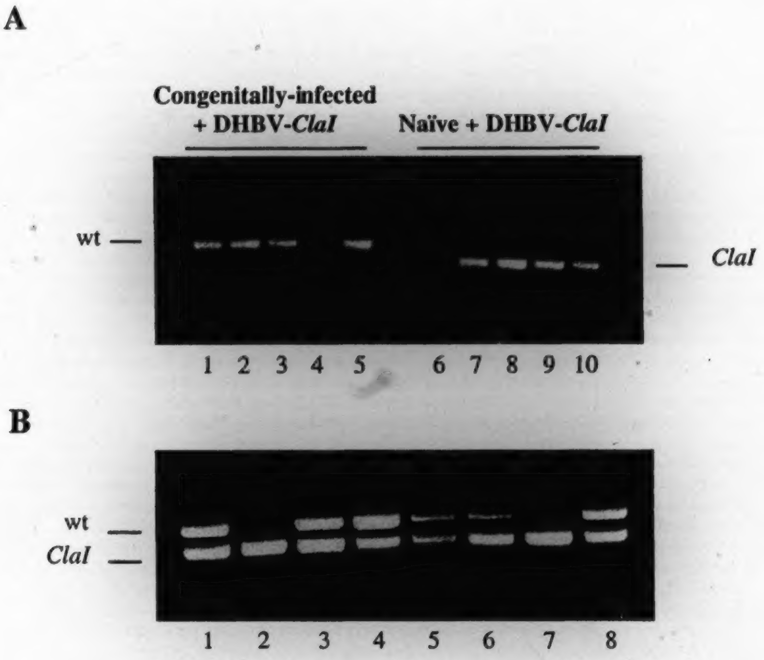

A
Congenitally-infected
+ DHBV-*ClaI*
Naïve + DHBV-*ClaI*
wt
ClaI
1 2 3 4 5 6 7 8 9 10
B
wt
ClaI
1 2 3 4 5 6 7 8

Figure 3.3: Infection of naïve and congenitally-infected ducklings with DHBV-*ClaI*. Ducklings were infected with 2 x 10^{10} VGE of either DHBV-*ClaI* alone or an mixture of DHBV-*ClaI*:DHBV. Serum viral DNA was extracted and amplified by PCR. The PCR products were digested with *ClaI* and analysed on an agarose gel. **A.** Five newborn congenitally-infected (lanes one to five) and five uninfected (lanes six to 10) ducklings were infected with DHBV-*ClaI*. Fourteen days post infection, serum viral DNA was analysed as described above. **B.** Eight newborn uninfected ducklings were infected with an equal mixture of DHBV-*ClaI* and DHBV. Results represent analysis of serum viral DNA seven days post-infection.

the virus present in the serum of the uninfected animals was entirely DHBV-*ClaI* (lanes six to ten), indicating that the DHBV-*ClaI* virus stock was infectious and that the *ClaI* digest was complete. Analysis of serum taken 21 days post-infection yielded the same result (data not shown). In total, 13 congenitally-infected and 12 uninfected animals were studied. All 12 uninfected animals were infected with DHBV-*ClaI* whereas none of the 13 congenitally-infected animals supported DHBV-*ClaI* infection. Four of the congenitally-infected ducklings were monitored for 12 weeks with no evidence of DHBV-*ClaI*. These results suggest that the pre-existing infection in these animals prevented superinfection by the DHBV-*ClaI* virus.

3.3.2 Co-infection of ducklings with DHBV and DHBV-*ClaI*. To determine if DHBV and DHBV-*ClaI* could establish a co-infection, a mixture of serum containing equivalent amounts of the two viruses was used to infect newborn ducklings. Eight one-day old ducklings were inoculated i.m. with 2 x 10^{10} VGE of a 1:1 mixture of DHBV and DHBV-*ClaI*. Serum viral DNA was analysed weekly as previously described and the results from seven days post-infection are shown in Figure 3.3b. Six of eight ducklings showed a mixture of both viruses in their serum. The remaining two ducks had predominantly DHBV-*ClaI* (lane two and seven). The results for 14 and 21 days yielded the same result. In addition, the ratios of DHBV and DHBV-*ClaI in* each duck remained consistent at the different time-points. In total, 13 of 18 ducks studied showed a co-infection with both viruses. The remaining five had predominantly either DHBV or DHBV-*ClaI*. The reason for the predominance of one virus or the other is not clear. The establishment of a co-infection in the majority of the animals indicates that simultaneous introduction of two viruses can result in the establishment of a dual infection. Thus, the lack of DHBV-*ClaI* in the congenitally-infected ducklings in the previous experiment was not due to a replicative advantage of DHBV over DHBV-*ClaI*.

Single-cell PCR was used to determine whether co-infections were the result of hepatocytes dually infected with both viruses or if DHBV and DHBV-*ClaI* replicated exclusively in separate cells. Newborn ducklings were infected with serum containing an approximately 1:1 ration of DHBV:DHBV-*ClaI* and monitored for co-infection by PCR of serum viral DNA. At two weeks post-infection, the liver of a co-infected duckling was perfused and the hepatocytes cultured for one week. The cells were then sorted into single cells by FACS (Figure 3.4) and viral DNA amplified and analysed as in previous experiments.

The results of the single-cell PCR analysis of the co-infected duck are shown in figure Figure 3.5b. The amount of PCR product from the hepatocytes of this animal was generally less than the PCR products from the animals used in the control experiment and so the viral DNA in these cells were analysed both by gel electrophoresis and Southern analysis. In total, 89/105 cells show the presence of both viruses. Five cells were infected exclusively with DHBV while 11 cells contained only DHBV-*ClaI*. Interestingly, analysis of serum viral DNA also showed slightly more DHBV-*ClaI* than DHBV. The results indicate that the majority of the cells contain both viruses.

To ensure that the cell sorting resulted in single cells and also that ECV did not contaminate the PCR, the livers of a congenitally-infected and a DHBV-*ClaI* infected duckling were perfused, cultured, and prepared separately as described. Prior to sorting, the cells were mixed in a 1:1 ratio. Because these cells are infected with either DHBV or DHBV-*ClaI*, single-cell PCR analysis should show only mono-infections in a single cell. The results of this control experiment are shown in figure 3.5a. In total, 48 out of 50 positive cells analyzed showed the presence of either DHBV of DHBV-*ClaI*, indicating that sorting does result in single cells and that any residual ECV remaining does not contaminate the PCR. The presence of both DHBV and DHBV-*ClaI* in the hepatocytes of the co-infected duck is therefore due to a dual infection and not cell doublets or ECV contamination.

3.3.3 Staggered co-infection of naïve ducklings with DHBV and DHBV-*ClaI*. To determine when the block to superinfection occurs, a staggered co-infection experiment was done. In this experiment, the two viruses were injected separately and the time between the two injections was varied. Fourteen one-day old naïve ducklings were inoculated i.m. with 1 x 10^{10} DHBV and then separated into seven groups of two ducks each. The first group received equivalent amounts of DHBV and DHBV-*ClaI* at the same time. Subsequent groups received equivalent amounts DHBV-*ClaI* one to six days

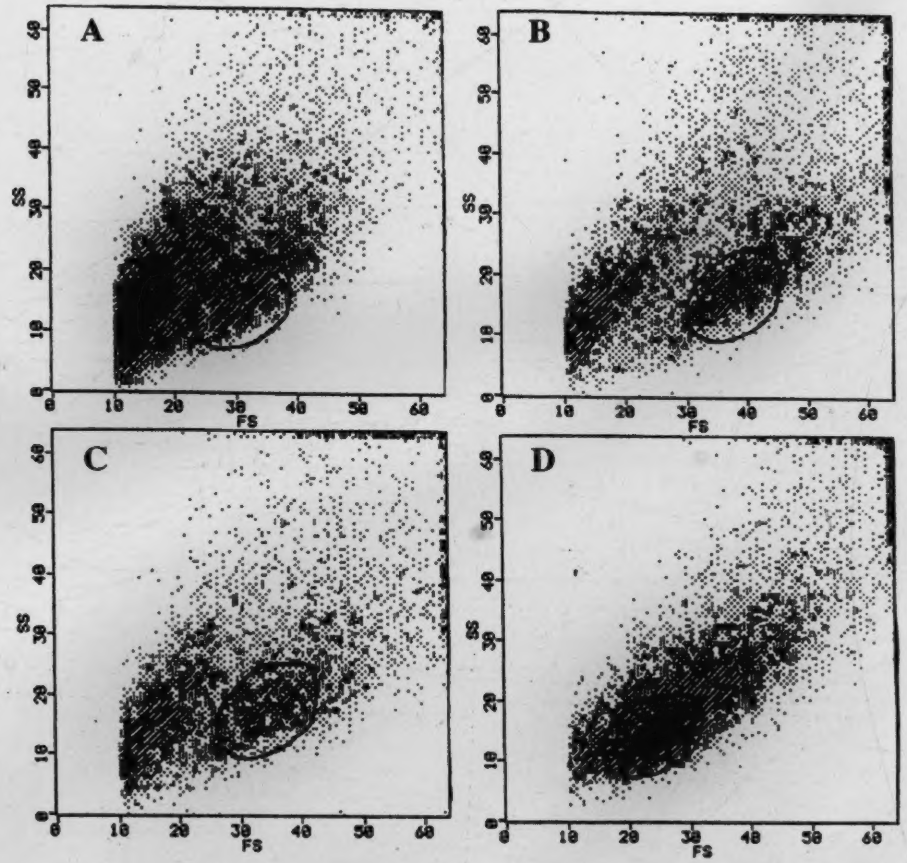


Figure 3.4: Sorting of primary duck hepatocytes into single cells. Primary duck hepatocytes were prepared and cultured as described. Three to five days post-plating the cells were washed 3 times with PBS, treated with glycine buffer pH 2.2 for 1.5 minutes to remove cell-bound virus, washed 2 times with PBS and then treated with trypsin. Cell viability was confirmed using Trypan blue prior to sorting. Figure represents FACS scans of cell suspensions. Circled area shows cell populations selected for sorting. **A.** DHBV-infected cells. **B.** DHBV-*ClaI*-infected cells. **C.** DHBV-infected and DHBV-*ClaI* infected cells mixed prior to sorting. **D.** Cells from a DHBV and DHBV-*ClaI* co-infected animal .

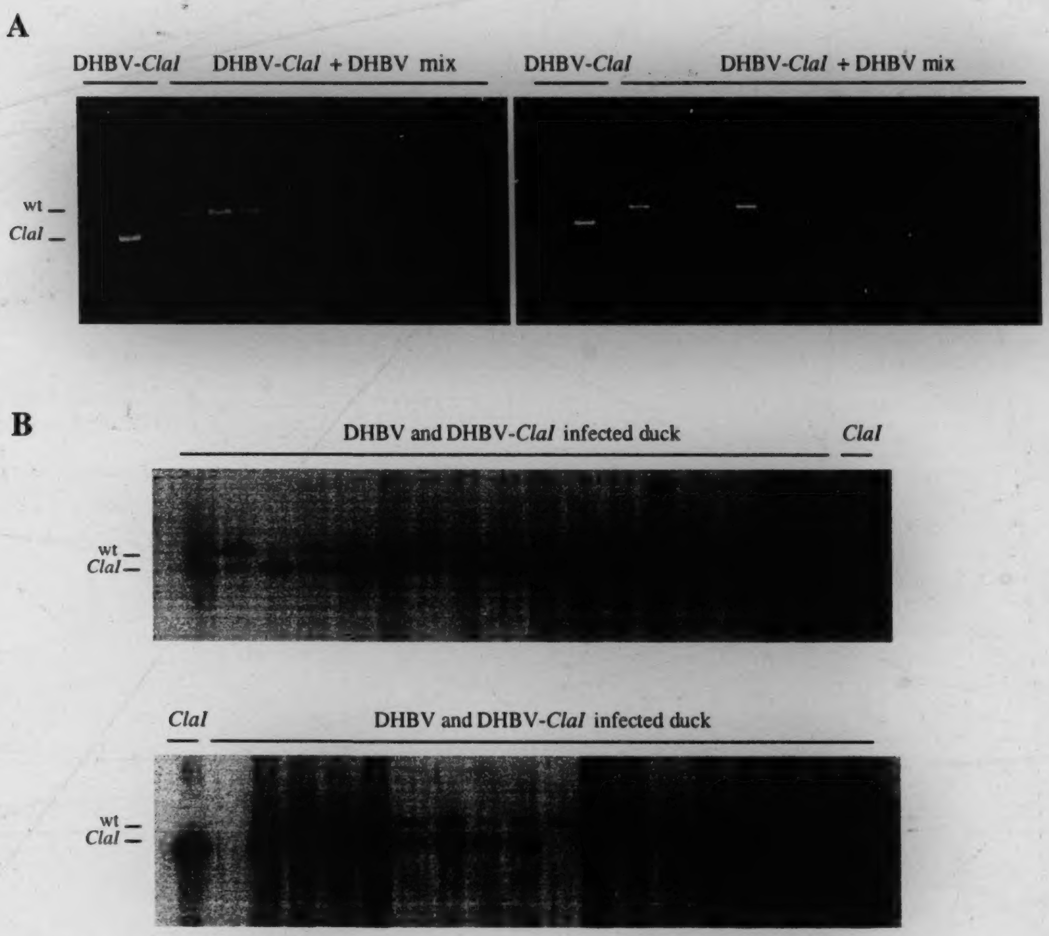

A
DHBV-*ClaI*
DHBV-*ClaI* + DHBV mix
DHBV-*ClaI*
DHBV-*ClaI* + DHBV mix
wt
ClaI
B
DHBV and DHBV-*ClaI* infected duck
ClaI
wt
ClaI
ClaI
DHBV and DHBV-*ClaI* infected duck
wt
ClaI

Figure 3.5: Single-cell PCR analysis of viral population in DHBV-infected hepatocytes. **A.** PCR analysis of individual hepatocytes from a DHBV-infected duck and a DHBV-*ClaI* infected duck that were mixed prior to sorting into single cells. PCR products were separated by size on a 1.2% agarose gel and visualised by Ethidium bromide staining. **B.** PCR analysis of individual hepatocytes from a DHBV:DHBV-*ClaI* co-infected duck. PCR products were separated by size on a1.2% agarose gel, transferred to a nylon membrane by Southern blotting and probed with a ^{32}P-labelled plasmid containing DHBV sequences. *ClaI* lanes represent *ClaI*-digest controls.

after they were infected with the original DHBV. Fourteen days after the DHBV-*ClaI* infection, the nature of the serum virus population was analysed by PCR and *ClaI* digestion. The results are shown in Figure 3.6a. Co-infection was seen when both viruses were administered simultaneously (lanes one and two) or when DHBV-*ClaI* was administered one (lanes three and four) or two days (lanes five and six) after the inoculation with DHBV. However, the groups that received DHBV-*ClaI* three or more days after the initial infection with DHBV showed only the presence of DHBV in their serum (lanes seven to 14). This experiment was completed three times and in all experiments the exclusion of the superinfecting virus occurred between two and three days. As with the previous experiment, there was variation in the ratio of DHBV to DHBV-*ClaI* in ducks that established a co-infection. These results indicate that exclusion of the superinfecting virus occurs as rapidly as three days after the initial infection.

To ensure that the failure to detect DHBV-*ClaI* at the later time points was not due to an age-related decrease of susceptibility to infection (9, 32), five naïve animals were infected with DHBV-*ClaI* at eight days of age. This was the last time-point at which DHBV-*ClaI* was introduced in the staggered co-infection experiment. All the animals were successfully infected with DHBV-*ClaI*, indicating that one week- old ducklings were still susceptible to infection (Figure 3.6b). Therefore, the absence of DHBV-*ClaI* in animals that received DHBV-*ClaI* three or more days after DHBV was likely the result of superinfection exclusion and not an age-related inability of the ducklings to become infected.

3.3.4 A lamivudine-resistant virus, DHBV-M512V, is unable to efficiently establish an infection in congenitally-infected animals despite having a selective advantage.

To assess whether a virus with a replicative advantage over the endogenous virus could establish an infection, DHBV-M512V was administered to congenitally-infected ducklings that were treated with lamivudine. DHBV-M512V contains the substitution of a valine for a methionine at the conserved tyrosine- methionine-aspartate-aspartate (YMDD) motif of the viral polymerase (7). This substitution is associated with resistance to the nucleoside analogue lamivudine (1, 7). Both DHBV and HBV containing this mutation have been shown to replicate at a lower rate than wild-type virus (21, 25).

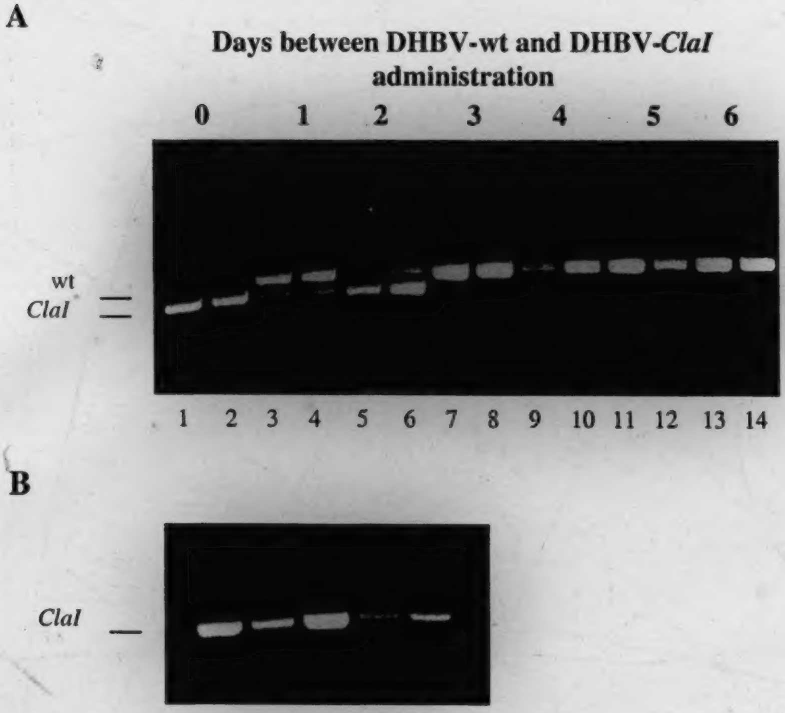

A
Days between DHBV-wt and DHBV-*ClaI* administration
0
1
2
3
4
5
6
wt
ClaI
1 2 3 4 5 6 7 8 9 10 11 12 13 14
B
ClaI

Figure 3.6: Staggered co-infection of ducklings. **A.** Fourteen newborn ducklings were infected with DHBV. They were then infected with DHBV-*ClaI* either the same day (lanes1 and 2) or one (lanes 3 and 4), two (lanes 5 and 6), three (lanes 7 and 8), four (lanes 9 and 10), five (lanes 11 and 12) or six (lanes 13 and 14) days later. Results represent analysis of serum viral DNA from each duckling 14 days post-DHBV-*ClaI* infection. Similar results are seen from samples taken 7 and 21 days after DHBV-*ClaI* infection. **B.** Five one-week old ducklings were infected with DHBV-*ClaI*. Results represent analysis of serum viral DNA one week post-infection.

However, because this mutation confers resistance to lamivudine, DHBV-M512V should still have a selective advantage over the endogenous virus in lamivudine-treated congenitally-infected ducks.

Fourteen congenitally-infected newborn ducklings were treated for one week with 40 mg/kg lamivudine i.m. twice daily to suppress levels of endogenous DHBV. Suppression of virus by lamivudine was confirmed by dot-blot (Figure 3.7). On day eight they were infected with approximately 5 x 10^9 VGE of DHBV-M512V. Ducklings were continued on lamivudine treatment to maintain suppression of endogenous DHBV and to prevent reversion of DHBV-M512V to wild-type DHBV. Serum samples were taken weekly, viral DNA isolated, amplified by PCR and the YMDD motif was analysed by DNA sequencing. Ten uninfected newborn ducklings of the same age were infected with DHBV-M512V and maintained on lamivudine therapy controls. The results of samples analysed for four weeks post-infection are depicted in Table 3.1. Twelve of the 14 congenitally-infected animals showed no evidence of DHBV-M512V superinfection as measured by PCR and sequencing. One duckling had only DHBV-M512V in its serum and another had a mixture of DHBV and DHBV-M512V. DHBV-M512V does not spontaneously arise in congenitally-infected ducklings maintained on long-term lamivudine therapy, even after two years of continuous therapy (Tyrrell, unpublished results). The presence of DHBV-M512V in the congenitally-infected ducklings is therefore almost certainly the result of the DHBV-M512V inoculation. Nine of the ten naive animals infected with DHBV-M512V were found to be positive for DHBV-M512V exclusively, indicating that the inoculum was infectious and that there was no reversion to wild-type DHBV. These results indicate that despite a selective advantage, DHBV-M512V was unable to establish an infection in the majority of congenitally-infected animals.

3.3.5 Exclusion of DHBV-*ClaI* in primary duck hepatocytes. To determine if superinfection exclusion could be observed *in vitro*, PDH from a congenitally-infected duckling were prepared and cultured as described. At three days post -plating, cells were washed twice with PBS and either mock infected (Figure 3.8a, lanes one to six) or

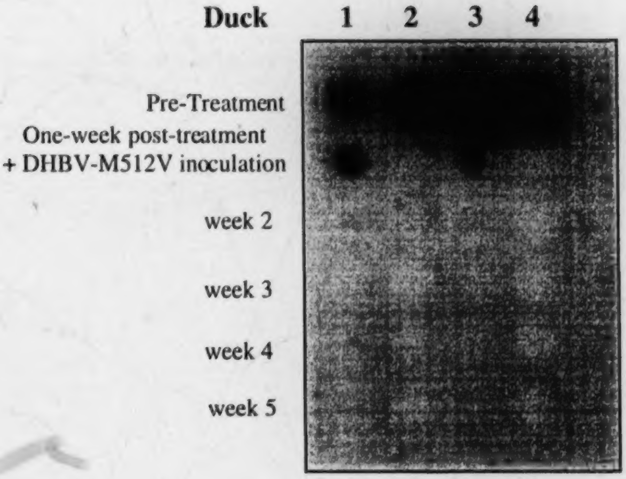
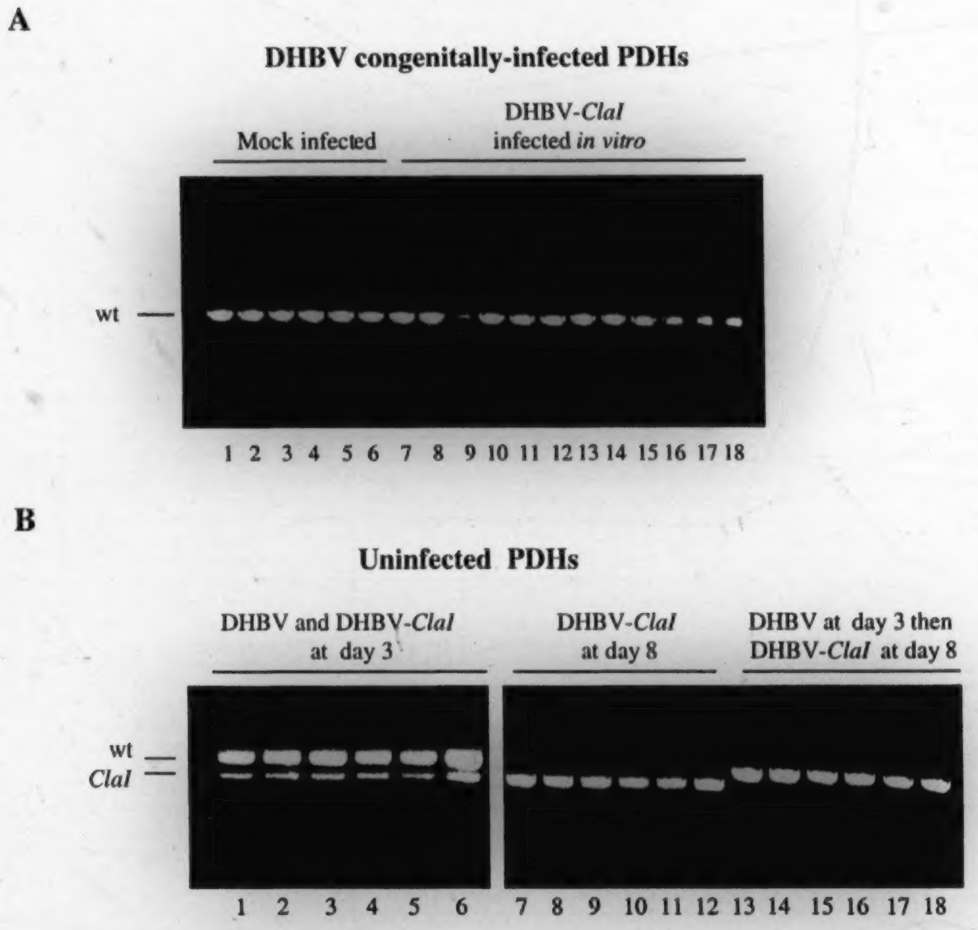

Duck
1
2
3
4
Pre-Treatment
One-week post-treatment
+ DHBV-M512V inoculation
week 2
week 3
week 4
week 5

Figure 3.7: Serum viral DNA analysis of congenitally-infected ducklings treated and lamivudine and infected with DHBV-M512V. Newborn congenitally-infected ducklings were treated for one week with 40 mg/kg lamivudine twice daily and then inoculated i.m. with DHBV-M512V. Animals were maintained on lamivudine therapy for the duration of the study. Results represent dot-blot analysis of serum viral DNA.

TABLE 3. 1. Inoculation of congenitally-DHBV-infected and naive ducklings with lamivudine-resistant DHBV-M512V

Sequence at codon 512 of DHBV polymerase	Congenitally-infected ducklings	Naive ducklings
ATG(wt)	12/14	0/10
GTG(M512V)	1/14	9/10
A/GTG(wt+M512V)	1/14	0/10

Fourteen newborn congenitally-DHBV-infected ducklings were treated for one week with 40 mg/kg lamivudine twice daily and then inoculated i.m. with DHBV-M512V. Ten naïve ducklings of the same age were similarly infected. All ducks were maintained on lamivudine therapy after inoculation with DHBV-M512V. Serum samples were taken weekly, viral DNA extracted and amplified by PCR. The PCR products were then sequenced to determine the sequence at codon 512 of the polymerase. Results represent weekly analysis of serum viral DNA for four weeks post-DHBV-M512V inoculation.



A
DHBV congenitally-infected PDHs
Mock infected
DHBV-*ClaI* infected *in vitro*
wt
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
B
Uninfected PDHs
DHBV and DHBV-*ClaI* at day 3
DHBV-*ClaI* at day 8
DHBV at day 3 then DHBV-*ClaI* at day 8
wt
ClaI
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3.8: Exclusion of DHBV-*ClaI* in PDHs. PDHs from congenitally-DHBV-infected or uninfected ducklings were harvested and plated as described. **A.** PDHs from a congenitally-DHBV-infected duckling were either mock-infected (lanes 1 to 6) or infected with DHBV-*ClaI* (lanes 7 to 18) three days post-plating. Intracellular viral DNA was harvested and analysed for the presence of DHBV-*ClaI* as described previously. Each lane represents intracellular viral DNA harvested from cells of one well of a six-well culture plate. **B.** PDHs from an uninfected duckling were either infected with DHBV and DHBV-*ClaI* simultaneously (lanes 1 to 6) at three days post-plating, infected with DHBV-*ClaI* alone at eight days post-plating (lanes 7 to 12), or infected with DHBV three days post-plating followed by infection with DHBV-*ClaI* at eight days post-plating (lanes 13-18). Intracellular viral DNA was harvested and analysed for the presence of DHBV-*ClaI*.

infected with DHBV-*ClaI* (lanes seven to eighteen) at a MOI of approximately 100. At 15 days post-plating, intracellular viral DNA was harvested, amplified by PCR and analysed for the presence of DHBV-*ClaI.* As shown in Figure 3.8a, no evidence of DHBV-*ClaI* was observed, suggesting that superinfection exclusion did occur *in vitro*.

It was important to distinguish whether the lack of DHBV-*ClaI* superinfection of congenitally-infected PDH was true exclusion or simply due to inefficient infection of the PDHs. To determine this, uninfected PDH were co-infected three days post-plating with a 1:1 mixture of DHBV and DHBV-*ClaI* at a MOI of 100-200. Fifteen days post-plating, intracellular viral DNA was harvested and analysed. A co-infection was seen in cells infected with both viruses simultaneously, indicating equal infectivity of both viruses (Figure 3.8b lanes one to six). Interestingly, the variation in the ratio of DHBV and DHBV-*ClaI* that was seen in ducklings co-infected with the two viruses was not seen in the co-infected PDHs. This indicates that the variation seen in animals likely involves factors other than the actual infection of the hepatocytes.

Alternatively, at three days post-plating cells were infected with DHBV only, at a MOI of 100-200. Five days later the cells were washed twice with PBS and infected with DHBV-*ClaI at* MOI 100. Uninfected cells cultured for the same amount of time (8 days) were also infected with DHBV-*ClaI* to ensure that the PDH were still susceptible to infection. Intracellular virus was harvested as before and analysed for the presence of DHBV-*ClaI.* DHBV-*ClaI* was detected in uninfected cells which were infected with DHBV-*ClaI* eight days post-plating (Figure 3.8b lanes seven to 12), but not in cells which had first been infected with DHBV three days post-plating and subsequently with DHBV-*ClaI* eight days post-plating (lanes 13 to 18). Southern analysis confirmed that virus present in cells infected with DHBV-*ClaI* only, 8 days post-plating, resulted from replicating virus, as indicated by the presence of the single-stranded form of viral DNA (Figure 3.9 lanes 7-12). Taken together, the results of these two experiments indicate that PDH are equally susceptible to infection with both viruses so the lack of DHBV-*ClaI* in the congenitally-infected PDH is unlikely due to inefficient infection by this strain. The lack of DHBV-*ClaI* in both the congenitally-infected PDH and naïve PDH infected with DHBV prior to DHBV-*ClaI is* therefore due to superinfection exclusion.

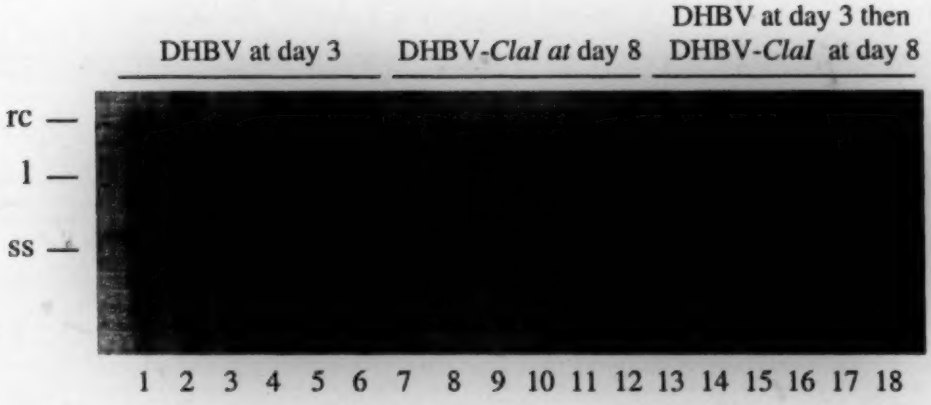

DHBV at day 3
DHBV-*ClaI at* day 8
DHBV at day 3 then DHBV-*ClaI* at day 8
rc
l
ss
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3.9: Southern analysis of intracellular virus from DHBV-infected PDHs showing active viral replication. PDHs from a naive duckling were either infected with DHBV three days post-plating (lanes 1-6), DHBV three days post-plating followed by DHBV-*ClaI* five days later (lanes 13-18) or DHBV-*ClaI* eight days post-plating (lanes 7-12). Intracellular viral DNA was harvested 15 days post-plating and analysed by Southern blotting. The probe consisted of a ^{32}P-labelled plasmid containing DHBV sequences. Each lane represents viral DNA from a well of a six-well culture plate. The positions of the replicative intermediates are indicated: relaxed-circular (rc), linear (l) and single-stranded (ss).

3.4 Discussion

The results of these studies show that an existing DHBV infection can prevent subsequent infection by a second hepadnavirus. Neither DHBV-*ClaI* nor DHBV-M512V was able to efficiently establish infections in congenitally-infected animals. Inoculation of uninfected ducklings with 10^5 VGE of DHBV resulted in an infection (data not shown). The amount of virus used to inoculate congenitally-infected animals, approximately 10^{10} VGE and 10^9 VGE for DHBV-*ClaI* and DHBV-M512V, respectively, was therefore 10,000-fold higher than the minimal titre required to establish an infection in ducks. It is significant, therefore, that no evidence of DHBV-*ClaI* infection could be detected in the congenitally-infected animals even after injection of high titre inoculum. Although the PCR assay is very sensitive, it is possible that a low level infection with DHBV-*ClaI* in the congenitally-infected animals occurred undetected. However, if this is true, it remains significant that the DHBV-infected hepatocytes in the congenitally-infected animals substantially reduced the ability of DHBV-*ClaI* to produce a vigorous infection in the liver.

Simultaneous inoculation of DHBV and DHBV-*ClaI* resulted in the establishment of a dual infection in naïve animals. Analysis of viral DNA present in individual hepatocytes indicates that the majority of cells in these ducks are infected with both viruses. Interestingly, some of the co-infected cells contained a predominance of one virus over the other. The variation in the ratio of the two viruses in co-infected cells may reflect the time between initial infection of the cell by each virus. For example, more DHBV in a co-infected cell could be the result of the cell first being infected by DHBV and then some time later by DHBV-*ClaI*. The fact that the majority of the cells are co-infected is perhaps not surprising, as exclusion of DHBV-*ClaI* in the staggered co-infection experiments was shown to take at least two days *in vivo*. If the mechanism of exclusion requires viral antigen expression there will be several hours of opportunity for the cell to be exposed to and infected with both DHBV and DHBV-*ClaI*.

The results of the staggered co-infection in *vivo* experiments indicated that exclusion occurred as rapidly as two days after infection with the first virus. A previous study by another group indicated that it takes approximately four days for the majority of hepatocytes to become infected when newborn ducklings are inoculated with DHBV

(10). It is possible that the time-point of exclusion seen in this study reflects the time it takes for the majority of hepatocytes to become infected with the first virus. During the first two days after infection with DHBV there may still be uninfected hepatocytes available for infection with DHBV-*ClaI*. Two or three days after infection with DHBV, however, the number of uninfected hepatocytes may be reduced to the point that it is difficult for DHBV-*ClaI* to establish an infection. The time it takes for all hepatocytes to become infected likely varies between animals due to liver size and titre of inoculating virus. Reducing or increasing the amount of the initial DHBV inoculum and determining the effect on the time of DHBV-*ClaI* exclusion would confirm this.

Congenitally-infected ducklings maintained on lamivudine and challenged with lamivudine-resistant DHBV-M512V resulted in only two of 14 animals showing evidence of DHBV-M512V, as measured by PCR. Again, a high titre of inoculating virus was used and shown to be infectious, as nine of ten uninfected animals injected became positive for DHBV-M512V. Lamivudine does not completely inhibit viral replication. Serum viral DNA is strongly suppressed as measured by dot blot but remains detectable by PCR in animals maintained on lamivudine therapy. However, DHBV-M512V should have a replicative advantage over DHBV in ducks maintained on lamivudine therapy and yet it was still unable to establish an infection in 12 of 14 animals. The presence of DHBV-M512V in the two remaining animals could be related to the number of DHBV-infected hepatocytes at the time they were infected with DHBV-M512V. Congenitally-infected animals vary in their level of viremia and a small percentage is able to clear the infection (Tyrrell, personal observation). This may explain why DHBV-M512V was able to establish an infection in two ducklings.

The significance of superinfection exclusion in hepadnavirus infections is unclear. It may be a mechanism by which the virus can limit viral replication to a level that is not cytopathic. Enhanced intracellular virus replication is associated with L surface antigen mutants that are defective in their ability to regulate cccDNA levels (15). The accumulation of higher levels of cccDNA has been shown to be cytopathic both *in vitro* and *in vivo* (14, 15). Superinfection exclusion might therefore be a consequence of the tight regulation of cccDNA levels, which in turn is necessary to maintain a non-cytopathic level of viral replication.

Exclusion could also explain the relatively slow emergence of lamivudine-resistant variants in chronic HBV patients treated with lamivudine. Previous studies have shown that enrichment of wild-type DHBV over a replicative-defective variant is rapid during the initial phase of infection when DHBV is spreading throughout the liver (35, 36). Once the majority of hepatocytes become infected, however, this enrichment of wild-type DHBV is much slower and appears to be dependent on an increase in liver mass. This implies that once a cell is infected by one virus, it cannot be infected by another and is consistent with superinfection exclusion. Therefore, the spread of any variant arising in a single cell, such as a lamivudine-resistant HBV, would be limited by the production of new uninfected hepatocytes or the total clearance of virus from infected cells. In a patient undergoing a liver transplant, the uninfected hepatocytes of the new liver would be susceptible to infection by the lamivudine-resistant HBV. Conversely, the liver of a chronically-infected individual undergoing lamivudine therapy still has a low level of wild-type viral replication and persistence of wild-type cccDNA, making it more difficult for the mutant to spread through the liver. This would explain why the development of lamivudine resistance is more rapid and occurs at higher rates in transplant patients compared with non-transplant patients.

Superinfection exclusion also has implications for proposed antiviral therapy using hepatitis B virus as a gene-therapy vector. Infection of congenitally-infected duck hepatocytes with a recombinant DHBV expressing green fluorescent protein (GFP) was significantly less efficient than infection of naive hepatocytes (24). In this study, more than 90% of non-infected hepatocytes were infected with the recombinant virus compared with 1-4% of congenitally-infected hepatocytes. Although this group did observe superinfection, it was extremely inefficient compared with infection of uninfected hepatocytes. The success of gene therapy for chronic HBV infection may therefore be limited by the ability of the recombinant HBV to enter an infected cell and express the therapeutic gene.

The mechanism of the observed superinfection exclusion is not known. Chronic hepatitis B carriers can be co-infected/superinfected with other hepatotropic viruses, including hepatitis A, C, and D viruses (5, 17, 18, 28) so the mechanism of superinfection exclusion likely involves HBV-specific factors. The results of both the single-cell PCR analysis of a co-infected duck and the staggered co-infection *in vivo* indicate that exclusion probably does not occur immediately after entry of the first virus. Furthermore, mechanisms of superinfection exclusion seen in other viral systems generally require some viral gene expression (4, 11, 34).

Two possible mechanisms of exclusion both involve the action of the L surface antigen. Firstly, expression of L surface antigen could affect the ability of the second virus to enter infected hepatocytes. Recently, it was shown that one of the putative receptors for DHBV, carboxypeptidase D, is down-regulated in a DHBV- infected hepatocyte (3). Breiner *et al* have shown that L surface antigen binds to carboxypeptidase D in the endoplasmic reticulum, causing premature degradation of the receptor. Exclusion of a second virus could be explained if the level of receptor down-regulation in an infected cell is sufficient to prevent viral entry. Receptor down-regulation as a mechanism of superinfection exclusion is seen in a number of viral infections, including HIV infections. The receptor, CD4, is down-regulated at translational and post-translational levels by the envelope, Vpu and Nef proteins (2).

Secondly, L surface antigen could also affect the establishment of the cccDNA pool of any superinfecting hepadnavirus virus. Establishment of the cccDNA pool occurs early in infection and is regulated by the L surface antigen (16, 26). Early in infection, when L surface antigen levels are low, nucleocapsids containing newly synthesised rcDNA are re-directed to the nucleus where the rcDNA is converted to cccDNA. As the cccDNA pool grows, the amount of L surface antigen increases and the nucleocapsids are enveloped and exported from the cell as infectious virus. Infected hepatocytes may have sufficient levels of L surface antigen that effectively block the amplification of cccDNA of the "superinfecting" virus. Without establishing a pool of its specific cccDNA, the second virus would not produce detectable extracellular virus. These two mechanisms are not mutually exclusive and may work together to prevent superinfection.

The pool of cccDNA is relatively resistant to the antiviral activity of lamivudine (22) and persists after prolonged lamivudine treatment (Tyrrell, unpublished results). Since cccDNA serves as the template for viral antigen transcripts, expression of the antigens should not be significantly reduced during the five weeks the congenitally-infected ducklings were maintained on lamivudine therapy (Tyrrell, unpublished results). Therefore, receptor down-regulation and/or inhibition of cccDNA amplification by the surface antigen of endogenous DHBV could explain the inability of both DHBV-*ClaI* and DHBV-M512V to superinfect the congenitally-infected animals. It also explains why superinfection exclusion can occur in the absence or reduction of viral replication. The next chapter deals with the investigation of the mechanism of exclusion mediated by hepadnaviruses.

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Chapter 4

Mechanism of Duck Hepatitis B Virus Superinfection Exclusion

4.1 Introduction

The results of Chapter 3 indicate that superinfection exclusion occurs in DHBV infection. The work in this chapter deals with the investigation of the mechanism employed by hepadnaviruses to inhibit superinfection. As mentioned in Chapter 3, a number of viruses are capable of mediating superinfection exclusion and a variety of mechanisms are employed. The most common mechanism involves the action of a viral transmembrane glycoprotein. Of these, that of HIV is perhaps the best studied. The down-modulation of the HIV receptor, CD4, renders cells resistant to superinfection (19). This is accomplished both through the action of Nef protein, which induces endocytosis of surface CD4 (1, 2, 13, 14), and the envelope glycoprotein, gp160, which complexes with CD4 inside the ER and prevents CD4 from reaching the cell surface (4, 10). Superinfecting virus is also inhibited in the absence of CD4 down-modulation by a mechanism that is not fully understood, but is dependent on the primary virus entering the cell and synthesizing DNA (31). In VSV infection, exclusion requires expression of the viral envelope glycoprotein G (32). Exclusion is caused by a sequence of effects on the endocytosis of superinfecting virus (25). First, there is a decreased production of endocytotic vesicles resulting in decreased internalization of receptor-bound virus. Secondly, there is competition between newly synthesized virus and superinfecting virus for occupancy of coated pits. Expression of the envelope glycoprotein of reticuloendotheliosis virus also mediates exclusion though hijacking its cellular receptor in the ER and preventing its display on the cell surface (12). Exclusion of HSV-1 is mediated by the viral glycoprotein D, which renders cells resistant to superinfection by inhibiting the adsorption of cell-bound virus particles (16).

Not all retroviruses mediate exclusion by down-regulation of their receptors. Human Foamy Virus mediates exclusion through the action of an accessory protein, Bet, which blocks replication of potential superinfecting viruses at a point between viral entry and provirus integration (3).

Inhibition of superinfecting Semliki virus is caused by a variety of mechanisms which inhibit viral RNA replication, entry and uncoating (26). The first, inhibition of viral RNA replication, occurs rapidly, within 15 minutes of infection by the primary virus. Other effects are seen at later times, including inhibition of virus binding, prevention of virus-endosome fusion which prevents release of the viral capsid into the cytoplasm, and blockage of nucleocapsid uncoating.

Vaccinia virus exclusion is independent of viral DNA replication but requires early gene viral transcription (9). The block in superinfection occurs at a point between virus adsorption and early gene transcription. The block in superinfection of cells persistently infected with Sindbis virus also occurs after attachment and entry of virus (17). The non-structural proteins of the superinfecting virus are translated but no viral RNA replication occurs. It is thought that the replicase required for synthesis of the (-) strand RNA, which serves as the template for viral RNA replication, is destroyed by the viral protease. Support for this comes from the fact that only nonstructural proteins, including the protease, are required for exclusion. In addition, a ts mutant of the protease is unable to mediate exclusion at non-permissive temperatures.

Resistance to superinfection in arenaviruses is observed with homotypic but not heterotypic viruses (11). It has been suggested that defective interfering particles mediate the exclusion by occupying viral receptors.

Since these mechanisms all require at least some gene expression of the primary virus, we investigated requirement for gene expression in hepadnavirus superinfection exclusion. As well, we attempted to identify the viral antigen which is capable of mediating exclusion and the point at which the block in superinfection occurs.

4.2 Materials and Methods

4.2.1 Cells, virus stocks and infections. Vero cells, an African green monkey kidney cell line (ATCC # CCL-81), were cultured at 37°C/ 5% CO_2 in MEM supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100μg/ml streptomycin and 50 IU/ml penicillin G and buffered with sodium bicarbonate. A human embryonal kidney cell line transformed with nucleotides 1-4344 of human adenovirus type 5 DNA, 293A, (ATCC# CRL-1573) were cultured at 37°C/5% CO_2 in DMEM supplemented with heat-inactivated FCS, 2mM L-glutamine, 100μg streptomycin and 50 IU/ml penicillin G and buffered with sodium bicarbonate. The human hepatoma cell line Huh7 was cultured in DMEM supplemented as described above. PDHs were prepared and cultured as described previously.

Dr. John Elliot (University of Alberta) kindly provided cell culture supernatant containing a recombinant adenovirus expressing a nuclear localized ß-galactosidase gene, Ad-ß-gal. Infections of PDHs were done 3-5 days post-plating at an MOI of approximately 5. PDHs were washed with PBS and incubated with 0.5-1ml of adenovirus-containing culture supernatant for 1 hour at 37°C. The supernatant was then removed and replaced with PDH medium. The number of adenovirus-infected cells was determined 24-48 hours post-infection by staining for ß-galactosidase activity. Briefly, cells were washed with PBS and fixed for 5 minutes at 4°C with 0.25% glutaraldehyde. Cells were then washed three times with PBS and incubated with 1ml of X-gal solution (1mg/ml X-gal, 2mM $MgCl_2$, 100mM each of potassium ferricyanide and potassium ferrocyanide in PBS) overnight at 37°C. Cells were washed twice with PBS and the number of ß-gal positive cells counted.

Dr. James Smiley (University of Alberta) kindly provided VSV. VSV was amplified by infecting near-confluent cultures of Vero cells at an MOI of less than 1. Virus-containing medium was incubated with cells for 1 hour at 37°C and then removed and fresh media added. Approximately 48 hours later cells were harvested, pelleted by centrifugation and resuspended in 0.5mls of serum-free MEM. Cells were subjected to three cycles of freeze-thaw conditions (alternating between dry ice/methanol bath and 37°C). Cell debris was removed by centrifugation and the virus stock stored in 100μl aliquots at -70°C. VSV stocks were titered by incubating Vero cell monolayers with serial dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}) of VSV stock at 37°C. After 1 hour, virus was removed and replaced with MEM media containing 1% methyl cellulose. Fourty-eight hours post-infection the MEM-methyl cellulose was removed, the cells were fixed and stained with Wright's solution (methanol andGiemsa stain) for 20 minutes and plaques counted.

For infection of PDHs, cell monolayers were incubated with culture media containing VSV at an MOI of 0.1-0.15 at 37°C overnight. VSV was then removed and replaced with fresh media. Cells were monitored visually for cytopathic effect daily. When the majority of cells were killed, the cells were washed with PBS, fixed and stained with Wright's solution.

Herpes simplex virus I (HSV) subtype KOS 1.1 was also provided by Dr. Smiley and used directly in infection studies. PDHs were infected three days post-plating with HSV at an MOI of 10 and were monitored daily for cytopathic effect.

4.2.2 Generation of recombinant adenovirus. Recombinant adenoviruses were generated using the AdEasy system. A schematic diagram depicting the general procedure is shown in Figure 4.1. Dr. Bert Vogelstein (Johns Hopkins Oncology Center) kindly provided the vectors pAdtrack-CMV and pAdEasy-1 and *E.coli* strain BJ5183.

A. Cloning of DHBV L surface antigen, S surface antigen, core, PreS/SΔ83-109 into the transfer vector pAdtrack-CMV. The nucleotide sequences encoding the L surface antigen, L surface antigen Δ83-109, S surface antigen and core genes of DHBV were first amplified using the Expand High Fidelity PCR System (Roche) and the following primers:

Gene	**Primer sequence**
L sAg:	Forward: 5'-CAGATATCACCATGGGGCAACATCCAGCAAAATCAATGG-3'
	Reverse: 5'-CAGATATCCTAACTCTTGTAAAAAAGAGC-3'
S sAg:	Forward: 5'-CAGATATCACCATGTCTGGTACCTTCGGG-3'
	Reverse: 5'-CAGATATCCTAACTCTTGTAAAAAAGAGC-3'
Core:	Forward: 5'- CTTGGGATCCGATGGATATCAATGCTTCTAGAGC-3
	Reverse: 5'-GCAAAGCTTTTATTTCCTAGGCGAGGGAG-3'

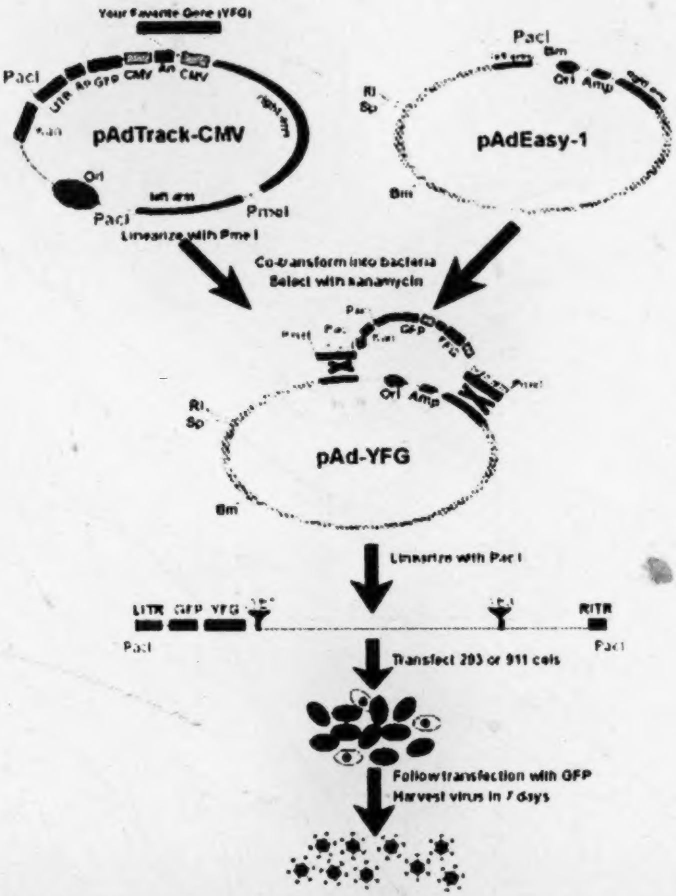

Your Favorite Gene (YFG)
PacI
LITR
CMV
An
GFP
pAdTrack-CMV
right arm
Kan
Ori
left arm
PmeI
Linearize with Pme I
PacI
pAdEasy-1
Bm
Ori
Amp
RI
Sp
Co-transform into bacteria
Select with kanamycin
pAd-YFG
Linearize with Pac I
LITR
GFP
YFG
RITR
Transfect 293 or 911 cells
Follow transfection with GFP
Harvest virus in 7 days

Figure 4.1: Schematic Outline of the AdEasy System. The gene of interest is first cloned into a shuttle vector pAdTrack-CMV. The resultant plasmid is linearised by digesting with restriction endonuclease *Pme I*, and subsequently cotransformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid, e.g. pAdEasy-1. Recombinants are selected for kanamycin resistance, and recombination confirmed by restriction endonuclease analyses. Finally, the linearised recombinant plasmid is transfected into adenovirus packaging cell lines, e.g. 293 cells. Recombinant adenoviruses are typically generated within 7 to 12 days. The "left arm" and "right arm" represent the regions mediating homologous recombination between the shuttle vector and the adenoviral backbone vector. An: polyadenylation site; Bm: BamHI, RI: EcoRI; LITR: left-hand ITR and packaging signal; RITR: right-hand ITR; Sp: SpeI.

http://www.coloncancer.org/adeasy/protocol2.htm

L sAg 1-82: Forward: 5'-CAGATATCACCATGGGGCAACATCCAGCAAAATCAATGG-3'
Reverse: 5'-CTCTTGAGGAGTCGGATTTGATAATCC-3'
L sAg 110-324: Forward: 5'- CGACTCCTCAAGAGGAAACCA
CCACCATTCCTCCGTCTTCC-3'
Reverse: 5'-CAGATATCCTAACTCTTGTAAAAAAGAGC-3'

Each 50 μl PCR reaction contained 100 ng of pCMV-DHBV, 1x Expand HF buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 12.5 pmol of each primer and 2.6 units of HF enzyme mix. Cycles for the PCR were as follows: 95°C for 4 minutes, then 15 cycles of [95°C for 30s, 60°C for 30 s, 72°C for 1 minute] followed by 72°C for 7 minutes. The L surface antigen PCR product was digested with *EcoR V* and cloned directly into *EcoRV*-digested pAdtrack-CMV to generate Adtrack-CMV-L surface antigen. The core PCR product was first blunt-ended, kinased and then cloned into *EcoRV*-digested pAdtrack to generate pAdtrack-CMV-core. To generate L surface antigen Δ83-109, fragments containing nucleotides 801 to 1047 and nucleotides 1131 to 1788 were amplified separately as described. The forward primer used to amplify amino acids 110-324 contains a sequence at the 5' terminus which is complementary to the reverse primer used to amplify amino acids 1-82. As a result, the 110-324 PCR product contains a 14-base overhang at the 5'end which allows it to anneal to the 1-82 PCR product. The PCR products were gel purified and used in a second PCR reaction with the same primers used to amplify full-length L surface antigen. The conditions of the second PCR reaction were 1x Expand HF buffer, 2.5 mM $MgCl_2$, 200 μM dNTPs, 12.5 pmol of each primer and 2.6 units of HF enzyme mix. Cycles for the PCR were as follows: 95°C for 4 minutes, then 20 cycles of [95°C for 30 s, 50°C for 30 s, 72°C for 1 minute] followed by 72°C for 7 minutes. The first 10 cycles were done in the absence of primers. L surface antigenΔ83-109 and S surface antigen PCR products were first sub-cloned into pCR2.1 (Invitrogen). pCR2.1-L surface antigen Δ83-109 and pCR2.1-S surface antigen were then digested with *EcoR V* and the resulting fragments cloned into *EcoR V*-digested pAdtrack-CMV to generate pAdtrack-CMV-L surface antigen Δ83-109 and pAdtrack-CMV-S surface antigen, respectively. A plasmid containing a 1.3-mer of the DHBV genome (constructed by Karl Fischer, University of Alberta) was digested with *Pst I/Sac I* to liberate the DHBV sequence. The resulting fragment was blunt-ended with T4 polymerase and cloned into *EcoR V*-digested pAdtrack-CMV to generate pAdtrack-CMV-DHBV. The integrity of the sequences generated by PCR amplification was confirmed by DNA sequencing using the primer 5'-GATCTGGTACCGTCGACGCGG-3'.

B. Generation of recombinants in *E.coli* BJ1583. Adtrack-CMV constructs were linearised by digestion with *PmeI*, followed by phenol/chloroform extraction and ethanol precipitation. Recombinants were generated by co-transforming electrocompetent *E.coli* BJ1583 cells with 100 ng each of the linearised Adtrack-CMV constructs and AdEasy-1. Electroporation was carried out in 20 µl cuvettes at 250V. Recombinants were screened for kanamycin resistance and recombination confirmed by restriction endonuclease analysis.

C. Recombinant adenovirus production and amplification in 293 cells. AdEasy-DHBV, L surface antigen, L surface antigen Δ83-109, S surface antigen, and core constructs were linearised with *PacI*, phenol/chloroform extracted and ethanol precipitated prior to transfections. T25 flasks of 50-70% confluent 293 cells were transfected with the linearised AdEasy constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, 5 µl of lipofectamine and 5 µg of DNA were used for each T25 flask of 293A cells. Ten days post-transfection, cells were harvested and subjected to four cycles of freeze/thaw in methanol/dry ice and a 37°C water bath with vortexing between cycles. Samples were spun and supernatant stored at -20°C. Approximately ½ of the supernatant from transfected cells was then used to infect additional T25 flasks of 293 cells. When one third to one half of the cells were detached (2-3 days), the cells were harvested by scraping off remaining cells, pelled and subjected to four cycles of freeze/thaw as described above. Additional rounds of amplification were done using T75 flasks of 293 cells. At this point, virus stocks were titered by infecting 293 cells with various dilutions of virus and counting the number of GFP-positive cells 18 hours later. To obtain high-titer stocks of the recombinant adenoviruses, 20 to 25 T75 flasks were seeded at 80% confluency and were infected at a 5-10 MOI.

Two to three days post-infection, cells were harvested and subjected to 4 cycles of freeze-thawing as described above. Virus stocks of 10^8-10^9 PFU/ml were obtained.

4.2.3 Fluorescent labelling of DHBV stocks. DHBV virions from duck serum were first partially purifed using a 20 % sucrose cushion. Approximately 20-30 mls of DHBV-positive serum was layered over 5-6 mls of 20% sucrose and centrifuged at 24,000 K for 18 hours at 4° C. The resulting pellet was resuspended in 500 µl of PBS. and then an equal volume of 2x Na Borate Buffer (400 mM pH 8.5) and 20 µg of 5-(and-6)-carboxy-X-rhodamine (succinimidyl ester) were added and the mixture was incubated at room temperature for 1 hour. Unreacted rhodamine label was removed by chromotography a a PD-10 gel filtration column (BioRad). The rhodamine-labelled DHBV (approximately 1.5 mls) was then incubated at 4° C with 250 µl of Afi-gel Blue beads (BioRad) for 2 hours to remove any remaining serum albumin. The sample was centrifuged briefly to remove the Afi-Gel Blue beads. Serum from a naïve animal was similarily labeled as a control.

4.2.4 Binding studies and confocal microscopy. Uninfected and DHBV-infected PDHs were prepared as previously described. Cells were incubated with rhodamine-labelled DHBV (equivalent to approximately 50µl of serum) at either room temperature or 4°C for 5-6 hours. PDHs were incubated with either labelled DHBV alone or labelled DHBV and 100 ③M of the PreS peptide (^{85}QPQWTPEEDQKA^{96}). The cells were then washed a minimum of six times with PBS to remove unbound virus and then analysed by fluorescence microscopy. For confocal microscopy, PDHs were grown on glass coverslips and incubated with rhodamine-labelled DHBV for 18 hours at 37°C. The cells were then washed six times with PBS and the nuclei were stained using Hoescht's solution (500 ng/ml) for 5 minutes at room temperature. The coverslips were mounted on slides using 50% glycerol and the cells were analysed with a Zeiss LSM 5 confocal microscope.

4.2.5 Infection of PDH with recombinant adenovirus. Two-day old cultures of PDHs were incubated with the recombinant adenoviruses at an MOI of 50 at 37°C overnight.

Cells were then washed once with PBS and fresh media added. Two days post-infection the efficiency of infection was determined by fluorescence microscopy to detect GFP-expressing cells. Antigen expression within infected cells was analysed by Western blot (described in detail below). Four days post-adenovirus infection, PDHs were infected with DHBV as previously described. One week later intracellular virus was harvested and analysed by Southern blot (described in Chapter 3).

4.2.6 SDS-PAGE and Western blot analysis of viral antigens and CPD. Cells were washed with PBS, harvested from cell culture dishes using a cell scraper and resuspended in 100 µl of 6x loading buffer (125 mM Tris pH 6.8, 5 % SDS, 10 % β-mercaptoethanol, 15 % glycerol and 0.1 % bromophenol blue). Proteins were separated by size using either 8% or 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were then transferred to nitrocellulose using transfer buffer (200 mM glycine, 25 mM Tris, 20% methanol) and a semi-dry transfer apparatus (Tyler). Non-specific binding of protein was blocked using 5 % skim milk/PBS/0.1 % Tween 20 for 3 hours at room temperature. Primary antibodies were diluted in blocking solution as follows: polyclonal anti-duck CPD (1:2500), polyclonal anti-duck core (1:1000), monoclonal anti-duck L suface and S surface antigens (1:20), monoclonal anti-chicken actin (1:1000). Blots were incubated with the primary antibodies either overnight at 4°C or room temperature for 3 hours. Following 3 x 15 minute washes with 4 % skim milk/PBS/0.1 % Tween 20, the blots were incubated with horse-radish peroxidase(HRP)-conjugated secondary antibodies (either goat anti-mouse or goat anti-rabbit) diluted 1:5000 in blocking solution for one hour at room temperature. Blots were then washed and antigen detected using ECL (Amersham) according to the manufacturere's instructions. The anti-CPD antisera was kindly provided by Dr. Heinz Schaller (University of Heidelberg), the anti-core antisera was provided by Dr. Jesse Summers (University of New Mexico) and the anti-L and S antibodies were obtained from Dr. Pat Nakajima (Fox Chase Institute, Philidelphia). The HRP-goat-anti-mouse and HRP-goat-anti-rabbit antibodies were obtained from Cappel.

4.3 Results

4.3.1 Uninfected and DHBV-infected PDHs are equally susceptible to infection with adenovirus, vesicular stomatitis virus (VSV) and HSV-1. The susceptibilities of naïve and DHBV-infected PDHs to adenovirus and VSV infection were compared to determine if the superinfection exclusion was specific for DHBV. Cells were infected with an adenovirus expressing a nuclear-localised beta-galactosidase at an MOI of approximately 1. Two days post-infection the cells were stained for beta-galactosidase activity. The results, shown in Figure 4.2a, show that there is no significant difference in the number of adenovirus-infected cells in uninfected and DHBV-infected hepatocytes. The same experiment was carried using HSV-1. PDHs were incubated overnight with HSV-1 at an MOI of 10. Cytopathic effect (CPE), characterised by rounding of the majority of cells, was observed 24 hours post-infection followed by cell death 48 hours post-infection. Again, there was no visible difference between the CPE observed in DHBV-infected and uninfected hepatocytes (Figure 4.2b) indicating that DHBV does not effect the ability of HSV-1 to infect PDHs.

Studies in HBV-transgenic mice have shown that HBV replicates to slightly higher levels in IFN-γ (Type II IFN) and IFN-α/β (Type I IFN) receptor knockout mice (20). This suggests that these cytokines can inhibit HBV replication to some extent. DHBV replication has also been shown to be inhibited by both Type I and Type II interferons (23, 24). Interferon-mediated inhibition of an established DHBV infection is minimal. The effect is greatest when interferon is present before or at the time of infection. It is possible that infection with DHBV induces a low level of interferon expression which does not effect the replication of the established DHBV infection but which inhibits the establishment of a second infection, in this case DHBV-*ClaI*.

The response of VSV to DHBV infection was used to test this idea. VSV is extremely sensitive to both Type I and Type II duck interferons (23, 24). If a low level of Type I interferon is expressed in DHBV-infected hepatocytes, they should be protected against VSV-mediated lysis. To examine this, DHBV-infected and uninfected PDHs were infected with VSV at an MOI of 0.1 and monitored daily for cytopathic effects. The

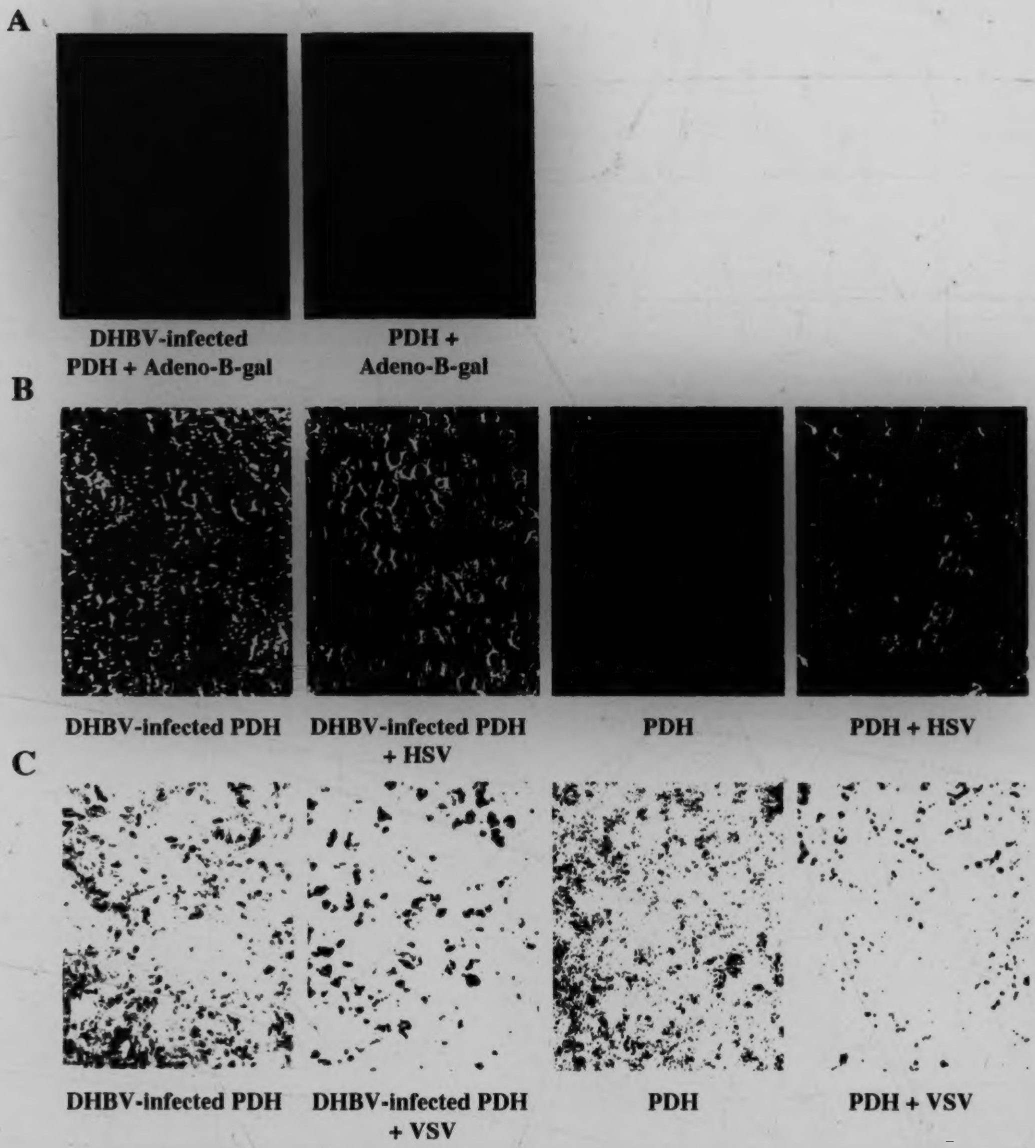

A
DHBV-infected PDH + Adeno-B-gal
PDH + Adeno-B-gal
B
DHBV-infected PDH
DHBV-infected PDH + HSV
PDH
PDH + HSV
C
DHBV-infected PDH
DHBV-infected PDH + VSV
PDH
PDH + VSV

Figure 4.2: Superinfection exclusion is limited to DHBV. Uninfected and DHBV-infected PDHs were prepared as described in Materials and Methods. **A.** Cells were infected with supernatant containing a recombinant adenovirus expressing B-galactosidase. Two days post-infection, cells were fixed and stained for B-galactosidase activity. **B.** Cells were infected with HSV-1 MOI 10 and monitored for cytopathic effect. Cells are shown 2 days post-HSV infection. **C.** Cells were infected with VSV MOI 0.1 and monitored for cytopathic effect. Cells are shown 4 days post-VSV infection.

majority of both uninfected and DHBV-infected hepatocytes were killed by day four post-infection, indicating that they were equally susceptible to VSV infection (Figure 4.2c). This suggests that the mechanism of DHBV exclusion is unlikely to be cytokine-mediated. The results from the adenovirus superinfection support this conclusion as the entry, and possibly the gene expression, of recombinant adenoviruses into cells has also been shown to be inhibited by interferon expression (20).

4.3.2 DHBV gene expression is required for exclusion of DHBV-*ClaI*. Mechanisms of superinfection exclusion in other viral infections, such as HIV, generally require at least some gene expression of the initial virus. The requirement for DHBV gene expression in DHBV-*ClaI* exclusion was examined by determining the effect of treating DHBV with UV-irradiation prior to infection of PDHs. UV-irradiation results in cross-linking of the viral DNA which inhibits transcription. Briefly, PDHs were infected with either DHBV, UV-treated DHBV (virus was exposed to UV for 1 hour at 4°C) or mock infected. The cells were harvested for Western analysis of viral antigens 3, 7, or 9 days later. At the same time-points, PDHs were infected with DHBV-*ClaI*. Intracellular virus was harvested one week after the last DHBV-*ClaI* infection (day 16) and analysed for the presence of DHBV-*ClaI*. The results are shown in Figure 4.3. As seen in Figure 4.3a, the amount of DHBV-*ClaI* seen in DHBV- infected cells, as compared to mock-infected cells, is decreased when DHBV-*ClaI* is added 3 days post-DHBV infection and almost completely absent when added 9 days post-DHBV infection. This indicates that DHBV-*ClaI* is excluded from cells that were first infected with DHBV. In contrast to this, DHBV-*ClaI* readily infected cells that were initially infected with UV-treated DHBV. The small amount of PCR product corresponding to the size of wild-type DHBV present in cells that were either mock infected or infected with UV-DHBV is likely due to incomplete digestion of the PCR product with the *ClaI* enzyme. Figure 4.3b shows the expression of the two envelope proteins, L and S surface antigen, from mock, UV-treated DHBV or DHBV- infected cells at the various times post-infection. The expression of both surface antigens can be detected in cells infected with DHBV. Neither of these antigens can be detected in cells infected with the UV-treated DHBV, indicating that this virus was incapable of gene expression. A small amount of each surface antigen can be

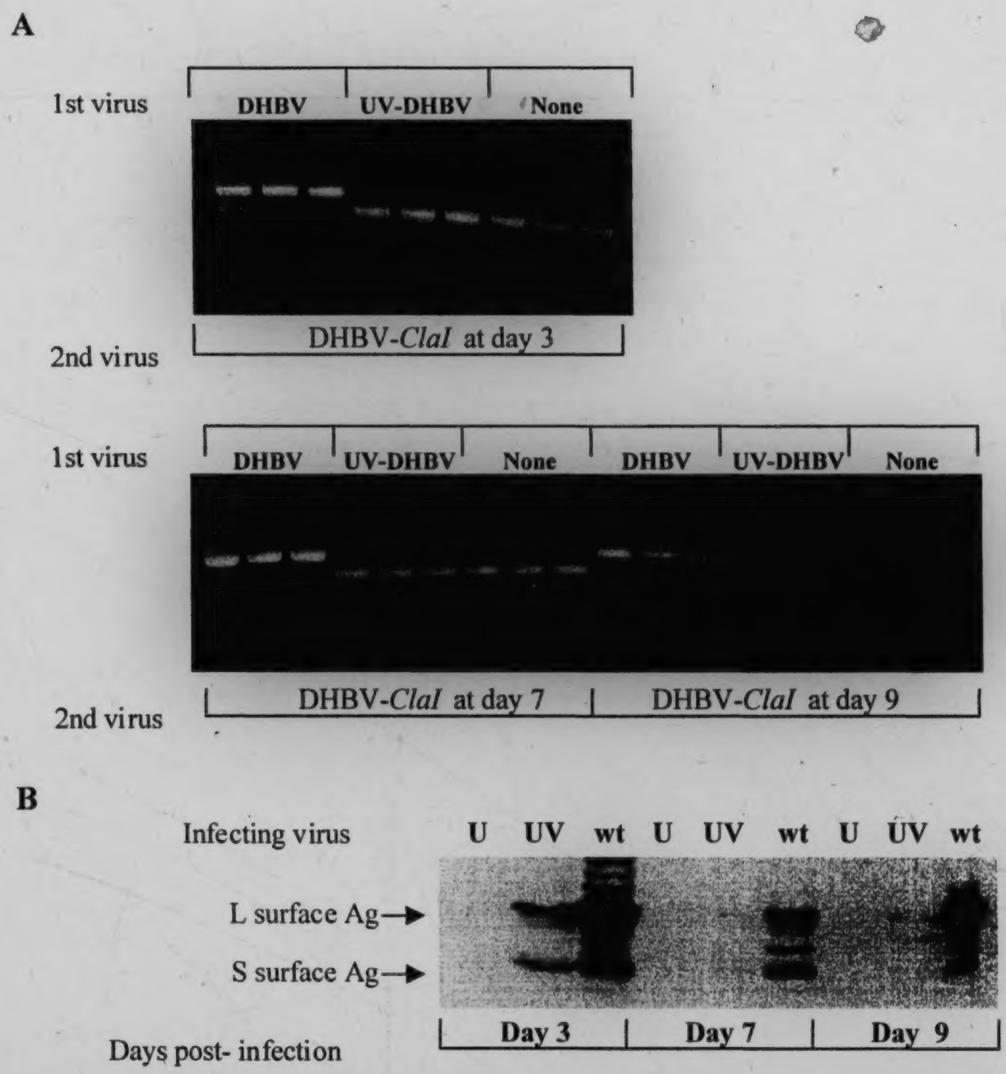

A
1st virus
DHBV
UV-DHBV
None
2nd virus
DHBV-*ClaI* at day 3
1st virus
DHBV
UV-DHBV
None
DHBV
UV-DHBV
None
2nd virus
DHBV-*ClaI* at day 7
DHBV-*ClaI* at day 9
B
Infecting virus
U
UV
wt
U
UV
wt
U
UV
wt
L surface Ag
S surface Ag
Days post- infection
Day 3
Day 7
Day 9

Figure 4.3. Superinfection exclusion of DHBV-*ClaI* is dependent on viral gene expression during the initial DHBV infection. A. PDHs were infected with either DHBV, UV-treated DHBV or mock infected. They were then infected with DHBV-*ClaI* either 3, 7, or 9 days later. Intracellular virus was harvested one week later and analysed for the presence of DHBV-*ClaI* as previously described. **B.** Uninfected cells (U) or cells infected with either UV-treated DHBV (UV) or DHBV (wt) were harvested and analysed by Western blotting for expression of large (L) and small (S) surface antigen.

detected three days post-infection but not at subsequent time-points and so likely represents the virus inoculum. Therefore, there does appear to be a correlation between the ability of DHBV to exclude DHBV-*ClaI* and viral antigen expression.

4.3.3 The identification of the DHBV antigen responsible for superinfection exclusion. The results of the previous experiment suggest that gene expression is required for exclusion of a second infection, in this case DHBV-*ClaI*. However, it does not indicate which viral antigen is involved. Knowledge of the viral antigen involved in the exclusion may provide some insight into its actual mechanism. Individual DHBV antigens were analysed for their ability to exclude DHBV infection to determine which one was responsible for the observed superinfection exclusion. As the transfection efficiency of PDHs is extremely inefficient (approximately 1-5%), recombinant adenoviruses were used to express DHBV antigens. Recombinant adenoviruses expressing DHBV core, L surface antigen, L surface antigen Δ 83-109, S surface antigen and a replication competent DHBV genome were constructed as described in the Materials and Methods. A schematic of the procedure is depicted in Figure 4.1.

Figure 4.4 demonstrates the expression of DHBV antigens in the recombinant adenovirus-infected PDHs. Cells were infected two days post-plating with the adenoviruses at an MOI of 100. Two days later the cells were harvested and analysed by Western blot analysis for the presence of the viral antigens. As seen in Figure 4.4a, the 36 kDa L surface antigen is present in cells infected with both Ad-L surface antigen and Ad-DHBV but not in cells infected with either the control adenovirus, Ad-GFP, Ad-S surface or Ad-core. The 17 kDa S surface antigen is present in cells infected with both Ad-S surface and Ad-DHBV but not in cells infected with Ad-GFP or Ad-core. A small amount of S surface antigen is present in cells infected with Ad-L surface. Cells infected with Ad-L surface Δ 83-109 express a truncated form of the L surface antigen. This is expected since it contains a deletion of amino acids 83-109, the region required for the interaction of L surface antigen with the viral receptor, CPD (30). Figure 4.4b shows the expression of the 31 kDa core protein in cells infected with either Ad-core or Ad-DHBV. The pattern of core expression in cells infected with Ad-DHBV is complex and likely

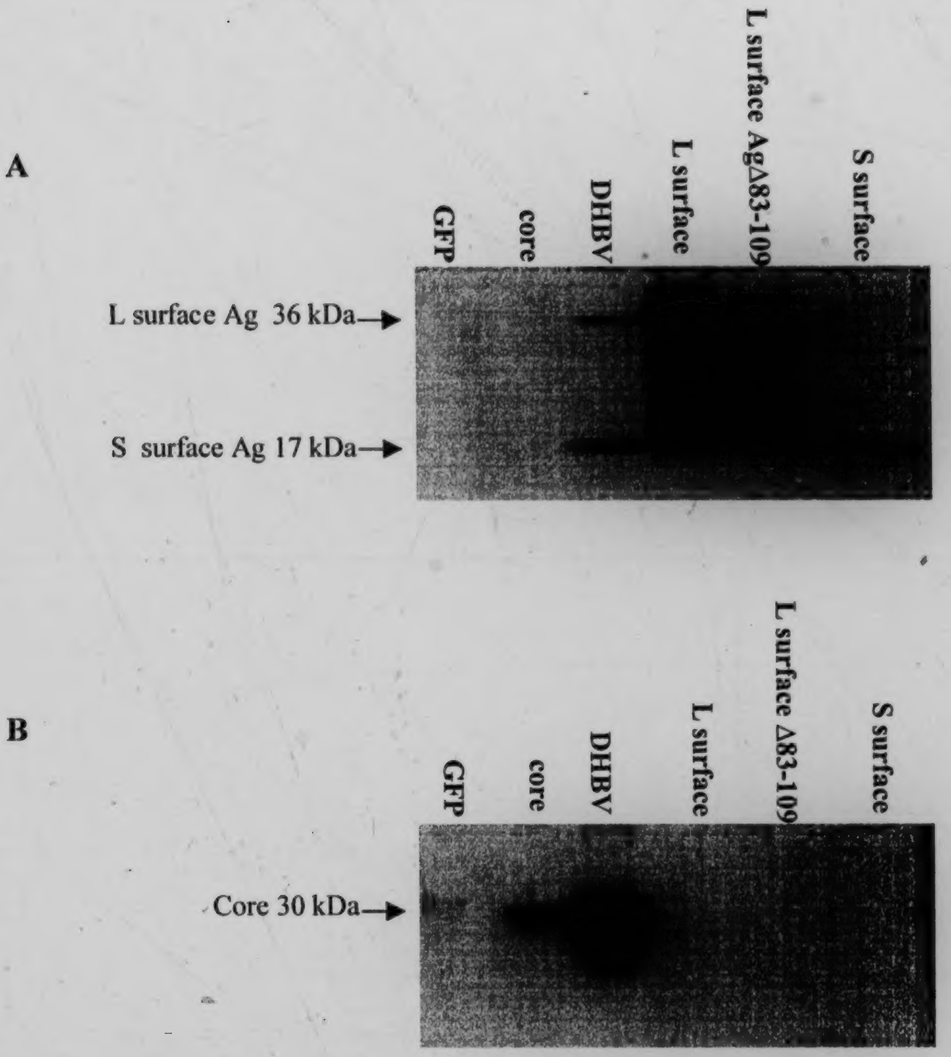

A
GFP
core
DHBV
L surface
L surface AgΔ83-109
S surface
L surface Ag 36 kDa
S surface Ag 17 kDa
B
GFP
core
DHBV
L surface
L surface Δ83-109
S surface
Core 30 kDa

Figure 4.4: Expression of DHBV antigens from recombinant adenoviruses. Naïve PDHs were infected with recombinant adenoviruses at an MOI of 100. Two days later the cells were harvested and analysed by Western blotting for the presence of either L and S surface antigens **(A)** or core antigen **(B)**.

represents the hyperphosphorylation of core protein from cytoplasmic nucleocapsids containing immature viral DNA.

Once efficient expression of viral antigens in PDHs infected with the recombinant adenovirus was confirmed, the ability of these recombinant adenoviruses to inhibit DHBV infection was investigated. PDHs were first infected with the the recombinant adenovirus at an MOI of 50. For these studies a lower MOI was used as a limited amount of cytotoxicity was observed with an MOI of 100 that was not apparent until approximately four days post-adenovirus infection. Four days post-infection the cells were analysed by fluoresence microscopy to determine the percent of cells infected by the recombinant adenovirus. In addition, some cells were harvested to confirm expression of the DHBV antigens. The adenovirus-infected PDHs were then infected with DHBV as previously described. One week later intracellular viral DNA was harvested and analysed by Southern blotting. As shown in Figure 4.5a, the efficiency of adenovirus infection (as measured by the percent of GFP-expressing cells) was about equal among the different adenoviruses, approximately 80%. This efficiency varied between experiments, ranging from approximately 60-80%. Evidence of DHBV infection, as indicated by the presence of the viral replicative intermediates, was seen in cells infected with Ad-GFP (Figure 4.5b, lanes 1 to 3), Ad-core (lanes 4 and 5) and Ad-S surface (lanes 12-14). Conversely, the level of DHBV infection was signficantly reduced in cells infected with either Ad-L surface (lanes 6 to 8) or Ad-L surface Δ 83-109 (lanes 9 to 11). This indicates that L surface antigen alone is capable of inhibiting DHBV infection of PDH. Figure 4.6a demonstrates the expression of the various DHBV antigens (core, L surface and L surface < 83-109 antigen, S surface antigen) in the adenovirus-infected cells at the time of DHBV infection (four days post-adenovirus infection). The expression of CPD is shown in Figure 4.6b. The expression of L surface antigen, but not the other antigens, resulted in a down-regulation of CPD. Western blot analysis of actin ensured equal loading of the lanes with cellular protein. Figure 4.7 and 4.8 represent repeats of the experiment illustrated in Figure 4.5. Occasionally, expression of the S surface antigen inhibited DHBV replication as compared to the control adenovirus, Ad-GFP, (Figure 4.8b, lanes 10-12). However, the extent of the inhibition was less than that seen with L surface and L surface <83-109 antigen.

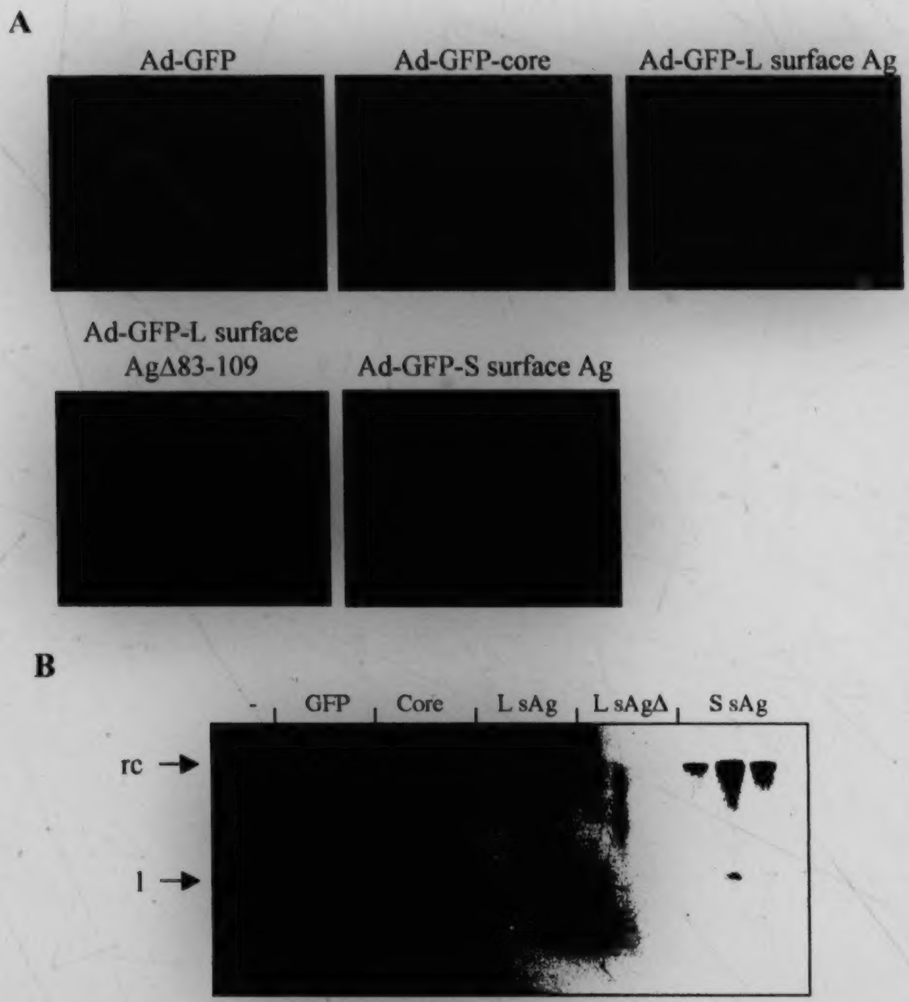

A
Ad-GFP
Ad-GFP-core
Ad-GFP-L surface Ag
Ad-GFP-L surface AgΔ83-109
Ad-GFP-S surface Ag
B
-
GFP
Core
L sAg
L sAgΔ
S sAg
rc
l

Figure 4.5: Superinfection exclusion is mediated by L surface antigen (Part I). A. Infection efficiency of PDH with recombinant adenoviruses. Cells were infected with recombinant adenovirus at an MOI of 50 two days post-plating. The number of infected cells was monitored by fluoresence microscopy. Results represent 4 days post-infection. Magnification, 10x. **B. Exclusion of DHBV by L surface Ag-expressing hepatocytes.** Cells were first infected with adenoviruses expressing either GFP alone (lanes 1-3), or GFP plus the DHBV antigens core (lanes 4-6), L surface Ag (lanes 7-9), L surface AgΔ83-109 (lanes 10-12), or S surface Ag (lanes 13-15). Four days later they were infected with DHBV. Results represent Southern blot analysis of intracellular virus 1 week post-DHBV infection. rc, relaxed-circular, l, linear.

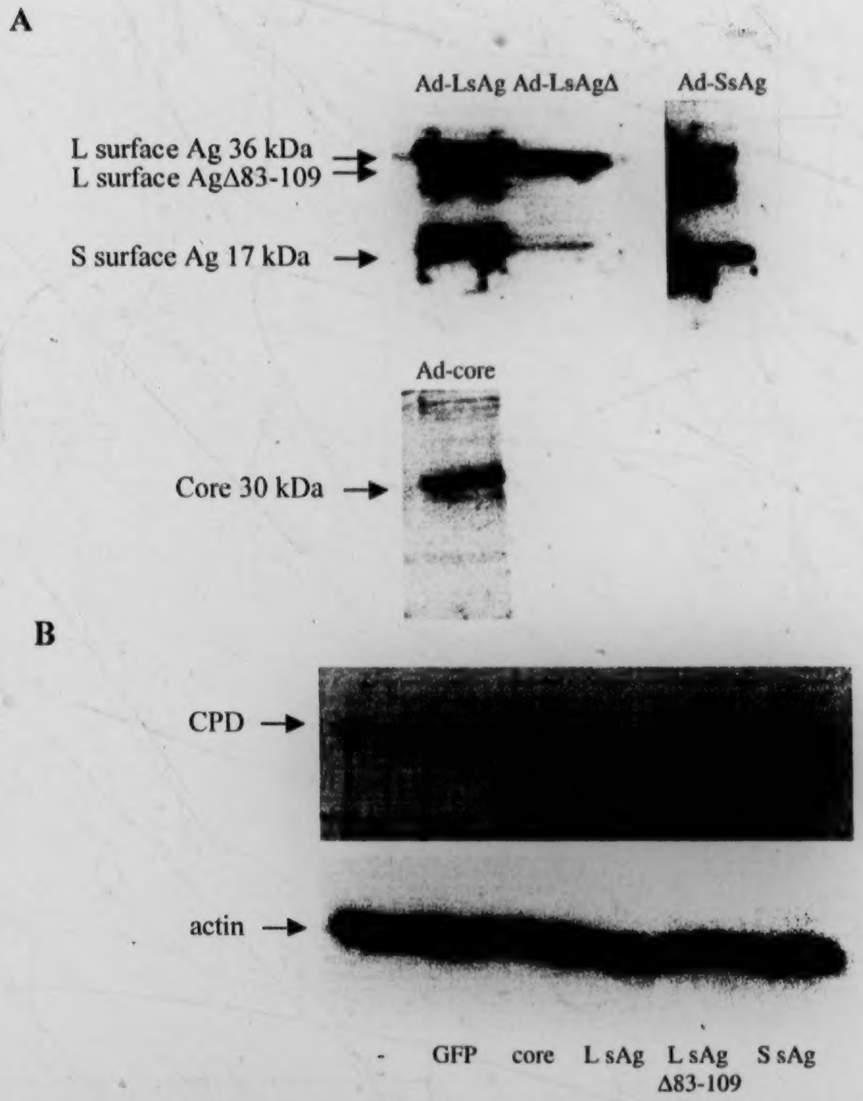

A
Ad-LsAg Ad-LsAgΔ
Ad-SsAg
L surface Ag 36 kDa
L surface AgΔ83-109
S surface Ag 17 kDa
Ad-core
Core 30 kDa
B
CPD
actin
-
GFP
core
L sAg
L sAg Δ83-109
S sAg

Figure 4.6: Western blot analysis of adenovirus-infected PDH. A. Expression of DHBV antigens in adenovirus-infected PDH. PDHs were infected with recombinant adenoviruses and examined by Western blot analysis for expression of DHBV antigens. **B. Expression of CPD and actin in PDHs infected with recombinant adenoviruses.** Uninfected PDHs or PDHs infected with adenovirus expressing either GFP alone, or GFP + core, L surface Ag, L surface Ag Δ83-109 or S surface Ag were analysed by Western blot for the expression of CPD (upper panel) on day 4 post-adenovirus infection. The same blot was stripped and probed for actin (lower panel).

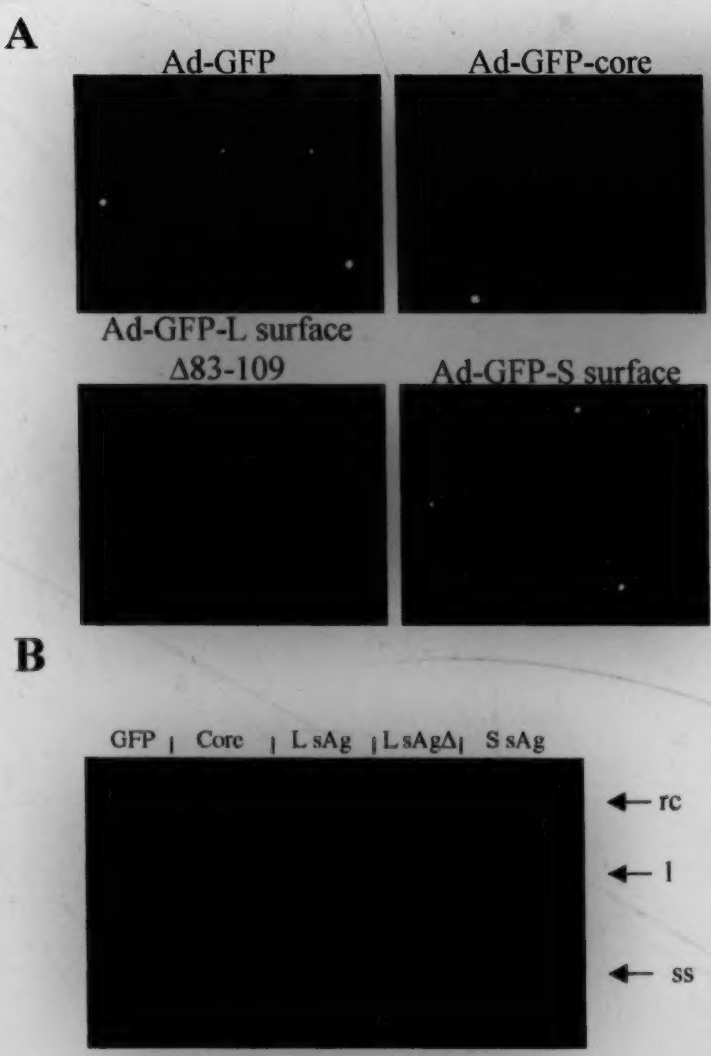

A
Ad-GFP
Ad-GFP-core
Ad-GFP-L surface
Ad-GFP-L surface Δ83-109
Ad-GFP-S surface
B
GFP
Core
L sAg
L sAgΔ
S sAg
rc
l
ss

Figure 4.7. Superinfection exclusion is mediated by L surface antigen (Part II). A. Infection efficiency of PDH with recombinant adenoviruses. Cells were infected with recombinant adenoviruses at an MOI of 50 two days post-plating. The percentages of infected cells were monitored by fluorescence microscopy. Results shown are 4 days post-adenovirus infection. Magnification, 40x. **B. Exclusion of DHBV by L surface antigen expressing hepatocytes.** Cells were first infected with adenoviruses expressing either GFP alone (lanes 1 and 2) or GFP plus the DHBV antigens: core (lanes 3-5), L surface antigen (lanes 6-8), L surface antigen Δ83-109 (lanes 9-11), or S surface antigen (lanes 12-14). Four days later they were infected with DHBV. Results represent a Southern blot analysis of intracellular virus one week post-DHBV infection. rc, relaxed-circular, l, double-stranded linear, ss, single-stranded.

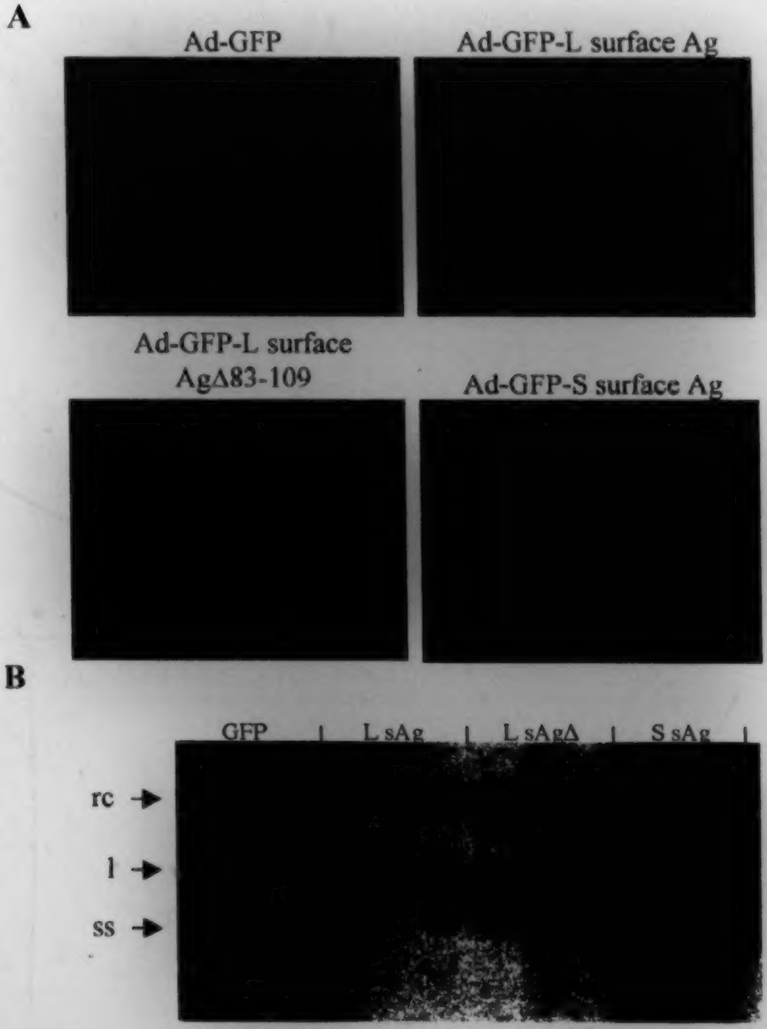
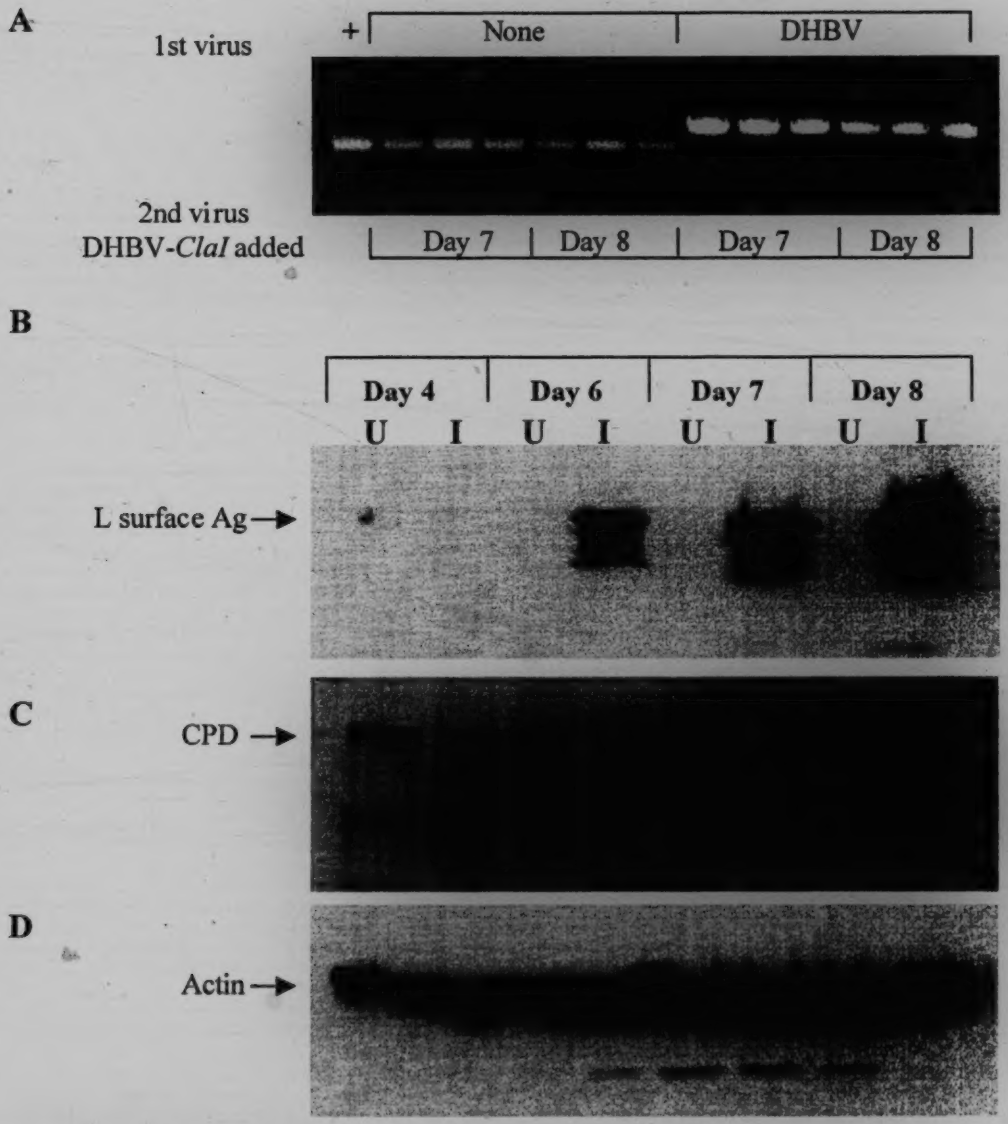

A
Ad-GFP
Ad-GFP-L surface Ag
Ad-GFP-L surface AgΔ83-109
Ad-GFP-S surface Ag
B
GFP
L sAg
L sAgΔ
S sAg
rc
l
ss

Figure 4.8: Superinfectin exclusion is mediated by L surface antigen (Part III). A. Infection efficiency of PDHs with recombinant adenoviruses. Cells were infected with recombinant adenoviruses at an MOI of 50 two days post-plating. The number of infected cells was monitored by fluorescence microscopy. Results are shown at 4 days post-adenovirus infection. Magnification, 40x. **B. Exclusion of DHBV by L surface Ag-expressing hepatocytes.** Cells were first infected with adenoviruses expressing either GFP alone (lanes 1-3), or GFP plus DHBV antigens: L surface Ag (lanes 4-6), L surface Ag Δ83-109 (lanes 7-9) or S surface Ag (lanes 10-12). Results represent a Southern blot analysis of intracellular virus 1 week post-DHBV infection. rc, relaxed circular. l, double-stranded linear. ss, single-stranded

4.2.3 A potential mechanism of L surface antigen-mediated exclusion of DHBV infection. L surface antigen was the only DHBV antigen that consistently inhibited DHBV infection and so likely it is involved in the mechanism of superinfection exclusion. As mentioned in both Chapters 1 and 3, L surface antigen down-regulates the DHBV receptor, CPD (6). The levels CPD were shown to decrease between 5 and 9 days post-DHBV infection. This is similar to the time at which DHBV-*ClaI* is excluded from DHBV-infected hepatocytes. As noted in the introduction, receptor down-regulation by viral envelope proteins is a mechanism of superinfection exclusion used by a number of viruses, most notably HIV, and so is a potential mechanism of DHBV exclusion. To test this, it was first determined if there was a correlation between DHBV-*ClaI* exclusion and a decrease in CPD expression. PDHs were first infected with DHBV and then 4, 6, 7, or 8 days later were either infected with DHBV-*ClaI* or harvested for Western blot analysis. In addition, naïve PDHs that had not been infected with DHBV were infected with DHBV-*ClaI* at these time-points. One week after the last DHBV-*ClaI* infection, intracellular virus was harvested and analysed by PCR as previously described. The expression of DHBV L surface antigen and core antigen was analysed by Western analysis. Figure 4.9a shows the PCR analysis of the intracellular viral DNA. In this experiment, partial exclusion of DHBV-*ClaI* was seen when DHBV-*ClaI* was introduced six days after the initial DHBV infection (data not shown) and was almost complete when introduced seven or eight days post-DHBV infection (Figure 4.9a). L surface antigen was first detected six days post-DHBV infection, correlating with the time when DHBV-*ClaI* exclusion was first apparant, with the expression levels steadily increasing (Figure 4.9b). However, the time-point of DHBV-*ClaI* exclusion did not correlate with a decrease in the expression of CPD (Figure 4.9c). An apparent decrease in CPD was observed 4 days post-infection, when superinfection was still observed, but returned to pre-infection levels by day 6. The presence of a faster-migrating band at this time-point which was absent in the remaining time-points indicates that this might be the result of degradation. Western analysis of actin ensured an equal quantity of cells in each lane (Figure 4.9d). Panels c and d represent the same blot stripped and re-blotted.



A
1st virus
+
None
DHBV
2nd virus
DHBV-*ClaI* added
Day 7
Day 8
Day 7
Day 8
B
Day 4
Day 6
Day 7
Day 8
U I U I U I U I
L surface Ag
C
CPD
D
Actin

Figure 4.9. Superinfection exclusion of DHBV-*ClaI* does not correlate with a decrease in carboxypeptidase D expression. A. Exclusion of DHBV-*ClaI* in DHBV-infected PDHs. PDHs were infected with DHBV. They were then infected with DHBV-*ClaI* 4, 6, 7, or 8 days later (only days 7 and 8 are depicted). Intracellular virus was harvested one week later and analysed for the presence of DHBV-*ClaI* using the PCR assay as previously described. Each lane represents viral DNA from a well of a six-well culture dish. **B, C, and D. Western blot analysis of DHBV-infected PDHs.** DHBV-infected (I) or uninfected (U) PDHs were harvested 4, 6, 7, and 8 days post-DHBV infection and the levels of L surface antigen **(B)**, carboxypeptidase D **(C)** or actin **(D)** analysed by Western blotting.

4.3.5 Binding of rhodamine-labelled virus to uninfected and DHBV-infected PDH.

Determining the cellular site at which superinfecting virus is blocked may provide clues to the mechanism of exclusion by L surface antigen. It is possible that L surface antigen interfers with the expression of a yet unknown co-receptor. Alternatively, it is possible that even though the total cellular expression of CPD is not affected at the time of exclusion, surface levels of CPD have decreased. If either of these is the case then the ability of DHBV-infected cells to bind labelled virus should be decreased compared with uninfected cells. Either DHBV-infected or uninfected PDHs were incubated with labelled virus or labelled control serum under conditions which have been shown to be permissive for DHBV-binding (five hours at room temperature) (22). After extensive washing with PBS, the cells were examined using fluorescence microscopy to detect cell-bound rhodamine-labelled virus. As seen in Figures 4.10b and 4.11b, the labelled virus was able to bind both uninfected and DHBV-infected hepatocytes, respectively. There was both a faint, but abundent fluorescence as well as an intense, particulate fluoresence. Incubation of cells with labelled control serum (serum from an uninfected duckling) did not result in either type of fluorescence on either uninfected or DHBV-infected hepatocytes (Figure 4.10 and 4.11a), indicating that the binding observed was specific to labelled DHBV and not labelled serum proteins. As well, labelled-DHBV did not bind to cell lines Huh7 and 239A, which are non-permissible for DHBV infection (data not shown). Incubating cells with labelled-DHBV in the presence of a peptide corresponding to the CPD binding domain of L surface antigen (amino acids 85-96 of the PreS domain) had no affect on the level of bound DHBV (Figure 4.10c and 4.11c). Although it was not possible to quantitate the amount of virus binding, it is clear that the virus is still capable of binding DHBV-infected hepatocytes. It is unlikely, therefore, that the block in DHBV infection of DHBV-infected hepatocytes is at the level of receptor binding.

4.3.6 Confocal microscopy analysis of uninfected and DHBV-infected hepatocytes incubated with rhodamine-labelled DHBV.

As the block in DHBV infection does not appear to be at the level of virus binding, we investigated the possiblity that entry of the virus by endocytosis is inhibited in DHBV-infected hepatocytes. Confocal microscopy was used to determine if rhodamine-labelled DHBV was able to enter hepatocytes.

A Control serum

B DHBV(+) serum

C DHBV(+) serum + PreS peptide

Figure 4.10: Binding of rhodamine-labelled DHBV to uninfected PDH. DHBV particles were partially purified from the serum of a DHBV-infected animal and then labelled with rhodamine as described in the Materials and Methods. Control serum from an uninfected animal was similarily treated and labelled. Cells were incubated with either rhodamine-labelled control serum (A), rhodamine-labelled DHBV (B), or rhodamine-labelled DHBV + a peptide corresponding to the CPD-binding region of PreS (C) for 5 hours at room temperature. The cells were then washed five times with PBS and analysed by fluorescence microscopy. Panels on the left were photographed using phase-contrast. Panels on the right were photographed using a filter specific for fluorescence.

A Control serum

B DHBV (+) serum

C DHBV (+) serum + PreS peptide

Figure 4.11: Binding of rhodamine-labelled DHBV to DHBV-infected PDH. DHBV particles were partially purified from the serum of a DHBV-infected animal and then labelled with rhodamine as described in the Materials and Methods. Control serum from an uninfected animal was similarily treated and labelled. Cells were incubated with either rhodamine-labelled control serum (A), rhodamine-labelled DHBV (B), or rhodamine-labelled DHBV + a peptide corresponding to the CPD-binding region of PreS (C) for 5 hours at room temperature. The cells were then washed five times with PBS and analysed by fluorescence microscopy. Panels on left were photographed using phase-contrast. Panels on right were photographed using a filter specific for fluorescence.

Rhodamine-labelled DHBV was incubated with DHBV-infected and uninfected PDHs at 37°C overnight. These conditions have previously been shown to be sufficient to allow DHBV entry into permissive cells. The cells were washed extensively with PBS and the nuclei were stained with Hoescht's dye. As seen in Figure 4.12, the virus was able to enter both uninfected (Figure 4.12a) and DHBV-infected (Figure 4.12b) hepatocytes. The labelled virus (red) and the nucleus (blue) are both in the same focal plane, indicating that the virus is within the cell and not simply bound to the cell surface. The fluorescent signal appears to be within endosomes in both the uninfected and DHBV-infected hepatocytes. However, whereas the endosomal-like membranes appear to be intact within the DHBV-infected hepatocytes, they appear to be disrupted within the uninfected hepatocytes. This may indicate that, in uninfected hepatocytes, the virus escapes from the endosomes while in the DHBV-infected hepatocytes the virus remains inside the endosomes. Incubation of uninfected and DHBV-infected hepatocytes with labelled control serum did not result in any binding as indicated by fluoresence microscopy and so these cells were not included in the confocal microscopy analysis (data not shown).

A Uninfected PDH

rhodamine
phase
Hoechst
combined

B DHBV-infected PDH

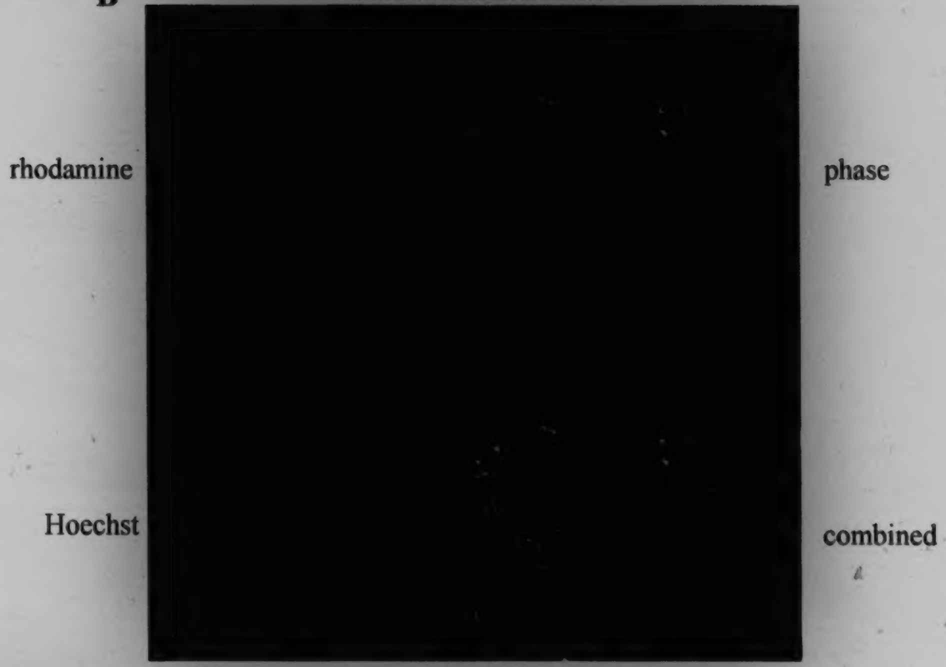

rhodamine
phase
Hoechst
combined

Figure 4.12: Confocal microscopy analysis of uninfected (A) and DHBV-infected (B) PDH incubated with rhodamine-labelled DHBV. Cells were incubated with rhodamine-labelled DHBV overnight at 37°C and then washed extensively with PBS. Nuclei were stained with Hoescht's prior to confocal microscopy analysis.

4.4 Discussion.

This chapter deals with the investigation of the mechanism of the superinfection exclusion observed in hepadnavirus infection described in Chapter 2. While the mechanism of exclusion was not elucidated, several important discoveries were made. First, exclusion is specific for hepadnaviruses. Unrelated viruses, ex. HSV I, adenovirus, and VSV were able to infect both naïve and DHBV-infected hepatocytes. As well, the exclusion is unlikely to be mediated by IFN since VSV-mediated killing was observed in DHBV-infected hepatocytes. Second, exclusion is dependent on viral gene expression. DHBV-*ClaI* was not excluded in cells infected with UV-treated DHBV. Third, exclusion of DHBV infection is dependent on expression of L surface antigen and possibly of S surface antigen. Fourth, exclusion of DHBV infection is not the result of decreased binding or entry of virus into hepatocytes.

Previous studies have indicated that the small number of liver-resident macrophages, Kupffer cells, that are present in primary duck hepatocyte cultures can be artificially stimulated by endotoxin to produce IFN (18). The level of IFN produced is sufficient to inhibit DHBV replication. However, the highly IFN-sensitive virus VSV was still capable of infecting and killing DHBV-infected hepatocytes. Therefore, the initial DHBV infection is unlikely to be producing sufficient IFN to mediate the exclusion of DHBV-*ClaI*.

Exclusion of DHBV-*ClaI* requires expresssion of viral gene products. UV-treated DHBV, which did not express viral antigens as shown by Western blot, was incapable of excluding DHBV-*ClaI* infection of hepatocytes. This is not surprising as the majority of viral interference mechanisms involves at least some viral gene expression. In addition, the results of the single-cell PCR analysis of infected hepatocytes from a DHBV-DHBV-*ClaI* infected duck described in Chapter 3 suggested that exclusion does not occur immediately after infection of a cell. As UV-treated virus should still bind to cells, this experiment indicates that viral interference is not likely mediated by the transient occupancy of cellular receptors by the initial virus, a mechanism of exclusion seen with retroviruses, including Rous sarcoma virus and Avian leukosis virus (27, 28).

Recombinant adenoviruses were used to individually express core, L surface antigen and S surface antigen in primary hepatocytes to determine which viral antigen mediates the exclusion. Expression of core protein did not inhibit DHBV infection compared with the control adenovirus, Ad-GFP, as indicated by comparable levels of DHBV replicative intermediates. However, expression of L surface antigen in hepatocytes, and occasionally to a lesser extent S surface antigen, resulted in a decreased level of DHBV replication. Again, this is not surprising. The envelope proteins of viruses are frequently involved in the mechanisms of superinfection exclusion. As well, the possible mechanisms of exclusion outlined in the Discussion of Chapter 3 involved the L surface antigen.

It is possible that the decreased level of DHBV replication in cells expressing the envelope proteins is due to cytopathic effects caused by overexpression of these proteins. However, while infection of primary hepatocytes with adenovirus does appear to have a limited cytopathic effect, the effects of all of the antigens were comparable and appeared to be related more to the MOI used in infection (the higher the MOI, the greater the cytotoxicity). Western blot analysis of actin levels at the time of DHBV infection of the adenovirus-infected hepatocytes showed no significant differences in cell numbers between the various antigens at the time of DHBV infection. Another group of investigators which expressed L surface antigen in PDH using recombinant adenoviruses at equivalent MOIs to these used in the present study did not report any cytotoxicity (6). Attempts to override L surface antigen-mediated down-regulation CPD by overexpression of of CPD failed, indicating that high levels of L surface antigen are present in the cell (6).

The inhibition of DHBV infection of PDH by L surface antigen also indicates that while expression of L surface antigen is required for exclusion of DHBV, viral replication is not. This observation is further supported by results obtained from an experiment described in Chapter 3 in which DHBV-M512V was used. In that experiment, the lamivudine-resistant DHBV- M512V was unable to establish an infection in the majority of congenitally-infected ducklings despite the suppression of wild-type virus replication, but not viral gene expression, with lamivudine. As well, the level of secreted virus present in the supernatant of infected primary hepatocytes at the time of DHBV-*ClaI* exclusion was virtually undetectable by PCR (data not shown). This is consistent with results obtained from other groups which show that levels of virus secreted from hepatocytes infected *in vitro* do not reach high levels until around 8-9 days post-infection (21, 29). It is unlikely, therefore, that exclusion of DHBV-*ClaI* is mediated by competition of DHBV-*ClaI* with L surface antigen or progeny virus for available cell surface receptors. In addition, L surface antigen, in the absence of S surface antigen, is retained intracellularly (15). While the L surface antigen construct contains the S surface antigen start AUG, very little S surface antigen is produced as indicated by Western analysis of cells infected with the Ad-L surface antigen. This is consistent with results from other groups of investigators and is likely due to inefficient initiation of translation of the internal S surface antigen AUG. While L and S surface antigen are encoded by the same ORF, they are expressed from separate mRNA transcripts (8).

Surprisingly, the L surface antigen-mediated down-regulation of the receptor CPD first demonstrated by Breiner *et al* and repeated in the present study does not appear to be involved in exclusion. This conclusion is based on a number of observations. First, the time of DHBV-*ClaI* exclusion in primary duck hepatocytes did not correlate with a decrease in CPD expression. DHBV-*ClaI* was excluded 5 to 7 days post-DHBV infection. At this time, Western blot analysis indicated that there was no significant decrease in total cellular CPD levels. However, it is possible that cell surface levels are decreased at this time. The use of cell fractionation to look specifically at the plasma membrane levels of CPD might give an indication of cell surface CPD levels. However, CPD is localised primarily to the Golgi apparatus and only transiently cycles to the cell surface and attempts to detect it on the cell surface of even uninfected hepatocytes is difficult (7).

Second, expression of a L surface antigen containing a deletion of the CPD-binding domain was still capable of excluding DHBV infection to levels comparable to wild-type L surface antigen. L surface antigen mediates down-regulation of CPD by interacting with CPD in the ER, leading to premature degradation of CPD (6). We hypothesised that deletion of the PreS domain involved in the L surface antigen-CPD interaction would eliminate the intracellular interaction of these two proteins and thus prevent CPD down-regulation. Western blot analysis confirmed that expression of L surface antigen Δ83-109 did not result in a down-regulation of CPD. Despite this, L surface antigen Δ 83-109 still inhibited DHBV infection.

Third, DHBV-infected hepatocytes were still capable of binding rhodamine-labelled DHBV. Conversely, rhodamine-labelled control serum did not result in any significant binding, indicating that the observed binding was specific for DHBV. As well, no binding was observed when the rhodamine-labelled DHBV was incubated with cells known to be non-permissive for DHBV infection. Further attempts to demonstrate specificity of virus binding by using the PreS peptide ^{85}QPQWTPEEDQKA^{96} failed. This region of PreS is crucial for the L surface antigen-CPD interaction (30) and it was thought that the peptide would bind cell surface CPD and block DHBV binding. However, the ability of this peptide to interact with CPD was not demonstrated in this study and it is possible that the peptide is too small to adopt a conformation required for the interaction (30). Alternatively, CPD may not be the sole DHBV binding receptor on the surface of hepatocytes (discussed below).

Labelled virus was also capable of entering DHBV-infected hepatocytes, apparently by endocytosis as indicated by the localisation of fluorescence in endosome-like vesicles. It is the surface of the virus which becomes labelled with rhodamine, most likely through attachment of the rhodamine to the L and S surface antigens present in the viral envelope. Fusion of the viral and endosomal membranes would disrupt the endosome membrane, allowing the release of the nucleocapsid into the cytoplasm. The fluorescent-labelled viral envelope would presumably remain associated with the disrupted endosome membrane. Interestingly, the margins of the endosome-like structures in the uninfected cells are not well defined, indicating that the virus may be exiting the endosome. Conversely, the margins of the virus-containing endosomes in DHBV-infected cells are well-defined. It is tempting to speculate that the block in superinfection is due to the inability of the superinfecting virus to escape the endosome.

While it is generally accepted that CPD is not the only receptor involved in DHBV entry, it is thought to be the primary cell surface receptor responsible for the attachment and endocytosis of DHBV into hepatocytes. However, the results of the present study question these conclusions. Labelled virus was still capable of binding and entering DHBV-infected hepatocytes. This is despite the fact that DHBV-*ClaI* is incapable of establishing an infection in these cells as shown in Chapter 3. While it has been shown that soluble CPD and anti-CPD antibodies block DHBV infection of hepatocytes *in vitro*, inhibition of binding to hepatocytes has not been investigated (30). It is possible that blocking the L surface antigen-CPD interaction blocks entry but not binding of DHBV to hepatocytes. However, this is unlikely as confocal microscopy demonstrated that DHBV is still capable of entering DHBV-infected hepatocytes. An alternative explanation is that very little CPD is required for efficient binding/entry of DHBV. It is unlikely that the down-regulation of CPD is absolute and so binding/entry of DHBV may be possible with a limited amount of cell surface CPD. The L surface antigen-CPD interaction has been shown to be unusually strong (30). Arresting CPD at the cell surface by deletion of the cytoplasmic tail has been shown to inhibit DHBV infection (5). This suggests that CPD is required for DHBV entry. However, the Breiner *et al* experiments were done using recombinant adenoviruses to express the mutant CPD which effectively outcompetes the endogenous wild-type CPD. Due to the strong affinity of L surface antigen for CPD (30), it is possible that the overexpression of the mutant CPD at the cell surface simply binds the virus and prevents it from interacting with additional co-factors which mediate the internalisation of virus.

In summary, superinfection exclusion in hepadnavirus infection is not IFN mediated. Exclusion requires viral gene expression but not viral replication. L surface antigen, and possibly S surface antigen, is capable of independently mediating exclusion. However, the block in superinfection occurs after attachment and internalisation of the virus into hepatocytes.

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Chapter 5

Summary and Future Considerations

5.1 Summary.

Lamivudine is a potent inhibitor of HBV replication (6, 7, 16) However, the effectiveness of lamivudine therapy for chronic HBV infection is limited by the selection of lamivudine-resistant variants (2-4, 7, 11, 20, 24). The first major theme of this thesis stemmed from the need to develop an *in vitro* assay system to identify/ screen for antiviral compounds active against the lamivudine-resistant variants. The second major theme of the thesis stemmed from unusual patterns of lamivudine resistance observed in clinical trials for lamivudine treatment of chronic HBV infection.

The *in vitro* assay system involved the production of stable cell lines expressing lamivudine-resistant HBV. The mutations in the HBV polymerase associated with lamivudine-resistance (2, 9, 11, 19-21, 23, 26, 29, 31) were introduced into replication-competent HBV constructs which were then stably transfected into a human hepatoma cell line. These cell lines produced both intracellular and extracellular virus which exhibited a marked decrease in sensitivity to lamivudine (2, 14, 15, 23). However, the results indicated that the resistant virus remained sensitive to two other nucleoside analogues, abacavir and CDG. This is promising for the possibility of developing combination therapy for chronic HBV.

Observations made during the initial clinical trials for lamivudine treatment of chronic HBV infection led to the speculation that superinfection exclusion occurs in hepadnaviral infections. Not surprisingly, lamivudine monotherapy results in the development of drug-resistant HBV variants. First, the average length of continuous treatment before the lamivudine-resistant mutants emerge is relatively long, around 8 months. Second, the establishment of the resistant virus appeared to be more efficient in patients who had undergone liver transplants. The emergence of resistance was more rapid and occurred at higher rates in transplant versus non-transplant patients. Recent studies using the DHBV animal model have also suggested that superinfection exclusion occurs in hepadnavirus infections. Studies of viral dynamics in ducks have shown that enrichment of wild-type DHBV over replication-defective mutants is rapid during the initial spread of infection (32). Thereafter, the enrichment rate is slower and appears to be dependent on the generation of new uninfected hepatocytes. As well, emergence of wild-type DHBV in competition studies with a cytopathic variant appears to be dependent on cell death caused by the mutant virus, as wild-type virus does not emerge in competition studies with a non-cytopathic variant (17).

The DHBV animal model was used to investigate the possibility of superinfection exclusion in hepadnavirus infection. The results in Chapter 3 indicate that superinfection exclusion does indeed occur in hepadnaviral infection. A genetically-tagged virus, DHBV-*ClaI*, was able to establish an infection in naïve but not congenitally-infected ducklings. However, simultaneous introduction of DHBV and DHBV-*ClaI* into naïve animals resulted in a co-infection with both viruses. Single-cell PCR analysis of hepatocytes from a co-infected duck indicated that the majority of the cells were infected with both viruses. If the time interval between the administration of DHBV and DHBV-*ClaI* into naïve animals was gradually increased, exclusion of DHBV-*ClaI* occurred as rapidly as two days after the initial DHBV infection. The results of both the staggered co-infection and single-cell PCR analysis of a co-infected duck suggest that exclusion does not occur immediately after the entry of the first virus. This is unlike the exclusion of Semliki virus in which exclusion of the superinfecting virus occurs as rapid as 15 minutes after the initial infection (25). Congenitally-infected ducklings were also treated with lamivudine to suppress DHBV replication and then challenged with a lamivudine-resistant DHBV, DHBV-M512V. Even though the resistant virus should have a selective advantage over the endogenous virus in the presence of lamivudine, the majority of the ducklings showed no evidence of infection with DHBV-M512V. Exclusion of DHBV-*ClaI* was also observed in vitro using primary duck hepatocytes. These results indicate that prior infection in animals excludes subsequent infection by a second hepadnavirus.

Chapter 4 deals with the investigation of the mechanism of hepadnavirus superinfection exclusion. Naïve and DHBV-infected primary duck hepatocytes are equally susceptible to infection with HSV-1, adenovirus, and VSV, indicating that exclusion is specific to hepadnaviruses. As well, due to VSV's extreme sensitivity to IFN, it is unlikely that the mechanism is interferon-mediated. Exclusion was found to be dependent on viral gene expression of the initial virus. More specifically, expression of L surface antigen alone was sufficient to inhibit DHBV of PDH. This result led to the assumption that exclusion was caused by the L surface antigen-mediated down-regulation of CPD (5). However, the time at which DHBV-*ClaI* exclusion occurred in DHBV-infected hepatocytes did not correlate with a decrease in CPD expression. A mutant L surface antigen which did not down-regulate CPD, or at least down-regulated CPD to a lesser extent than wild-type CPD, was still capable of mediating exclusion. As well, exclusion of DHBV-*ClaI* from infected hepatocytes is not at the level of virus attachment or entry. Rhodamine-labelled virus was still able to bind and enter DHBV-infected hepatocytes. In summary, while superinfection exclusion in hepadnavirus infections is mediated primarily by L surface antigen, the exact mechanism remains to be determined.

5.2 Directions for future work.

The generation of stable cell lines expressing lamivudine-resistant HBV provide an convenient system to screen antiviral compounds. These cell lines have already been used to study the effect of a group of novel anti-hepadnavirus compounds called alkovirs (22). Future work should include additional screening of potential antiviral compounds. The cell lines could also be used to further characterise YMDD motif mutants. For example, intracellular replicating cores could be isolated and used in enzymatic kinetic studies on the various mutants.

The results in this thesis indicate that while superinfection exclusion is mediated by the DHBV L surface antigen, the mechanism does not appear to be due to an inability of the superinfecting virus to bind or enter hepatocytes as a result of L surface antigen-mediated down-regulation of CPD (5). Another potential mechanism of L surface antigen-mediated exclusion was suggested in Chapter 3. L surface antigen is a negative regulator of cccDNA amplification which is essential for the establishment of hepadnaviral infections (18, 27, 28). L surface antigen mediates this inhibition by interacting with mature nucleocapsids, facilitating their export from the cell as enveloped virus which prevents the rcDNA from contributing to the cccDNA pool. Several single amino acid substitutions in the PreS domain of L surface antigen, including D128 and L131, prevent L surface antigen from regulating cccDNA pools as evidenced by the accumulation of abnormally high levels of cccDNA in cells infected with viruses containing these mutations (18). This is presumably because the cytosolic PreS domain is unable to interact with mature nucleocapsids. Mature nucleocapsids which would normally be targeted for secretion from the cell are instead transported to the nucleus where the rcDNA is converted to cccDNA. In future studies, these mutations should be introduced into the L surface antigen in the context of the recombinant adenoviruses and analysed for their ability to exclude DHBV infection. As well, a previously developed assay for quantitating DHBV cccDNA should be used to determine if cccDNA is produced in L surface antigen-expressing cells infected with DHBV (1).

Confocal analysis of primary hepatocytes incubated with rhodamine-labelled DHBV indicated that the block in superinfection occurs post-internalisation. It appears that exit of the virus from the endosomes may be inhibited. First, similar experiments done at 4°C and in the presence of endocytosis inhibitors should be done to confirm that the fluorescent vesicles are endosome-localised virus. As well, co-localisation experiments with endosome-specific markers would confirm that the virus is within endosomes. Second, time-course studies tracking the progress of rhodamine-labelled DHBV in uninfected and DHBV-infected hepatocytes may provide more information about when the block to superinfection is occuring. For example, time-points of 1 hr, 6 hrs, 12 hrs, and 24 hrs following the addition of labelled–DHBV to hepatocytes could be used to study the kinetics of viral entry in uninfected and DHBV-infected hepatocytes. Transmission electron microscopy might be an alternative way of tracking the virus following incubation with uninfected or DHBV-infected hepatocytes.

5.1 Significance of superinfection exclusion in viral infections.

The reasons for superinfection exclusion in viral infections are, for the most part, speculated upon rather than experimentally proven. It is interesting that the majority of viral interference systems studied involve enveloped viruses (HBV, HIV, HSV, VSV, vaccinia, Semliki). While this does not necessarily mean that only enveloped viruses are capable of mediating exclusion, it does suggest that for some reason they are more inclined to do so.

Superinfection exclusion may be a mechanism to ensure efficient spread and hence replication of the virus. Preventing re-infection of infected cells forces progeny virus to search out naïve cells, resulting in a greater number of infected cells. This may partially explain why establishing superinfection exclusion is more important for enveloped viruses. In general, non-enveloped viruses release progeny virus by lysing the infected cell. This ensures that progeny virus will go on to infect naïve cells. Conversely, enveloped viruses may exit cells in the absence of cell death, either by budding from the plasma membrane or exiting through the cellular secretory pathway. Without cell death there is no guarantee that progeny virus will spread to new target cells. Therefore, establishing viral interference may be more important for enveloped viruses to ensure efficient spread of the progeny virus. Further support for this idea comes from the observation that some enveloped viruses have evolved mechanisms to either destroy or down-regulate the expression of its cellular receptor. For example, both influenza and bovine coronavirus encode an enzyme which cleaves sialic acid residues off cell surface molecules (30). As both these viruses attach to cells via interaction with cell surface sialic acid residues, this cleavage effectively destroys the virus receptor. Measles virus down-regulates its cellular receptor, CD46, through the action of the hemagglutinin H protein (8, 10, 12). Down-regulation of CD46 by measles virus renders the cell susceptible to complement-mediated lysis. This may serve both to release progeny virus and, because the infected cell is killed, ensure that the progeny virus spreads to new target cells.

Superinfection exclusion may also be related to the regulation of virus replication levels within infected cells to prevent cytopathology. As mentioned in Chapter 3, exclusion in hepadnaviral infection may be a result of the the inhibition of cccDNA amplification by the L surface antigen. This regulation of cccDNA, the template for further viral replication, may be important in maintaining viral replication at a level that is not cytotoxic to the cell. Interestingly, superinfection exclusion in Sindbis virus infection may also be the result of regulation of viral replication. Cells persistently infected with Sindbis virus inhibit the viral RNA replication of superinfecting virus. It appears that synthesis of the template for RNA replication, the (-) sense RNA, is inhibited through the action of a viral protease which destroys the replicase complex (13). Primary infection of cells with Sindbis virus is associated with high levels of viral replication and severe cytopathic effect. Conversely, persistent infection of cells, which are resistant to superinfection with other alphaviruses, is associated with a low level of virus replication and little cytopathology.

Superinfection exclusion may be a mechanism to deal with the production of defective-interfering particles (DI) during viral infections. The genomes of DI particles frequently contain deletions and as a result are replicated much faster than the full-length genomes of infectious virions. Superinfection of cells with the faster replicating DI particles may result in a larger number of DI particles produced compared to infectious virions. Infectious virions would then have to compete with DI particles for infection of new target cells.

In summary, while the reasons for superinfection exclusion are not fully understood, it is a phenomenon that is conserved among a relatively wide variety of viruses. That fact alone suggests its significance as viruses have evolved to be extremely efficient at what they do best; replicate!

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Appendix 1-Plasmid Maps

pAdTrack-CMV-L surface Ag

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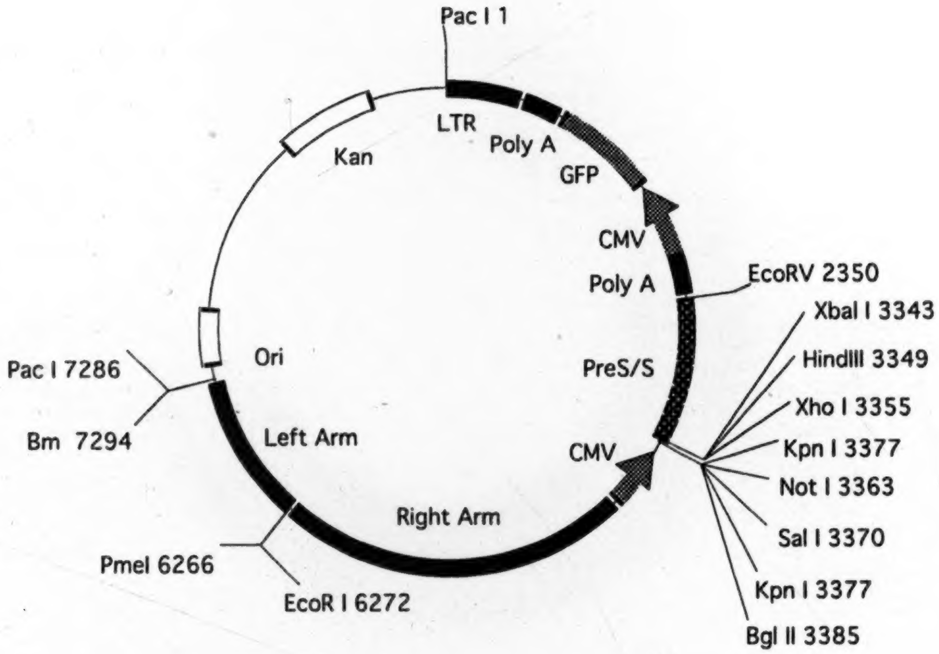
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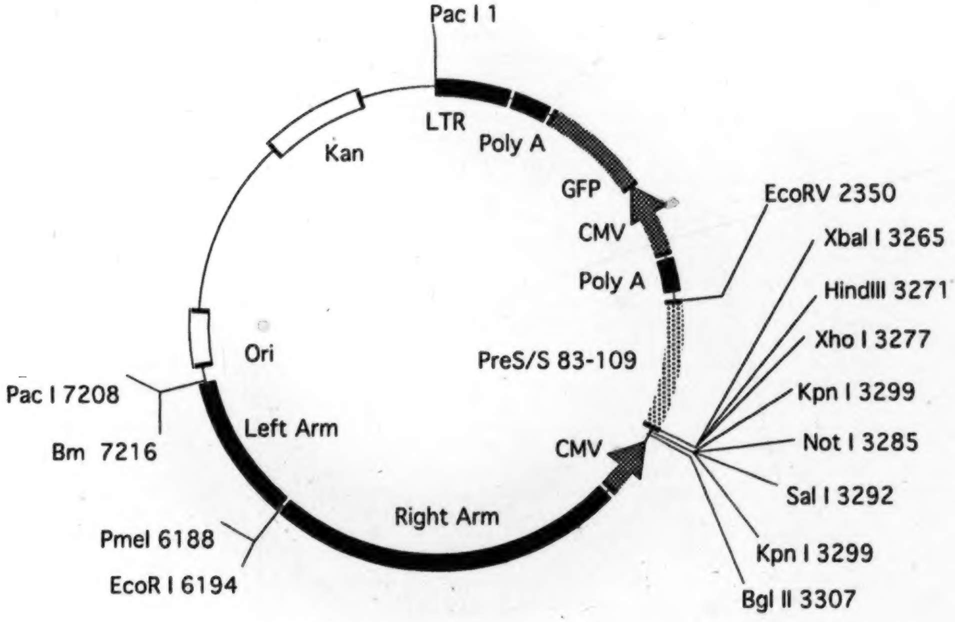
GFP
CMV
Poly A
EcoRV 2350
XbaI I 3343
HindIII 3349
Xho I 3355
Kpn I 3377
Not I 3363
Sal I 3370
Kpn I 3377
Bgl II 3385
PreS/S
CMV
Right Arm
Left Arm
EcoR I 6272
PmeI 6266
Bm 7294
Pac I 7286
Ori
Kan

pAdTrack-CMV-L surface Ag del83-109

10129 bp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Poly A
Kan
GFP
CMV
Poly A
EcoRV 2350
XbaI I 3265
HindIII 3271
Xho I 3277
Kpn I 3299
Not I 3285
Sal I 3292
Kpn I 3299
Bgl II 3307
PreS/S 83-109
CMV
Ori
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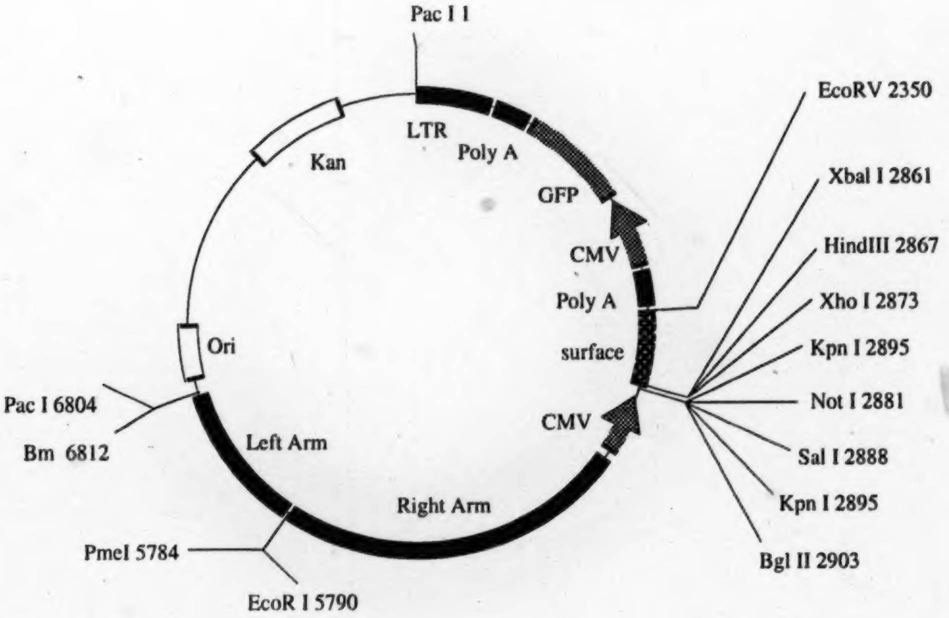
Bm 7216
Left Arm
Right Arm
PmeI 6188
EcoR I 6194

pAdTrack-CMV-S surface Ag

9725 bp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LTR
Poly A
GFP
CMV
Poly A
surface
CMV
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Left Arm
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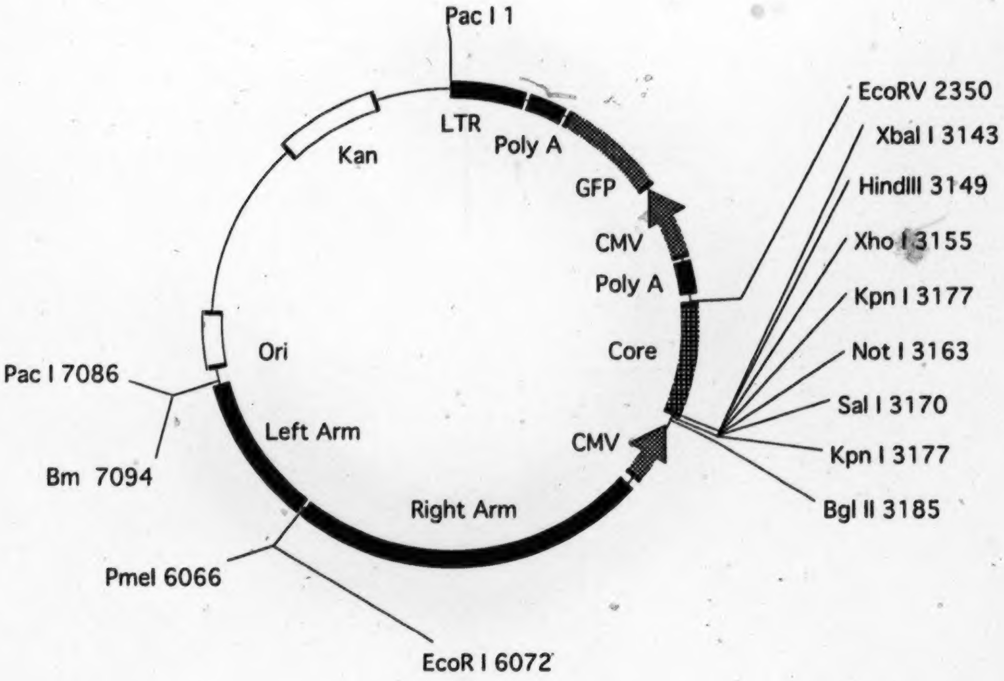
pAdTrack CMV-core

10007 bp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Left Arm
Right Arm
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Xho I 3155
Kpn I 3177
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Sal I 3170
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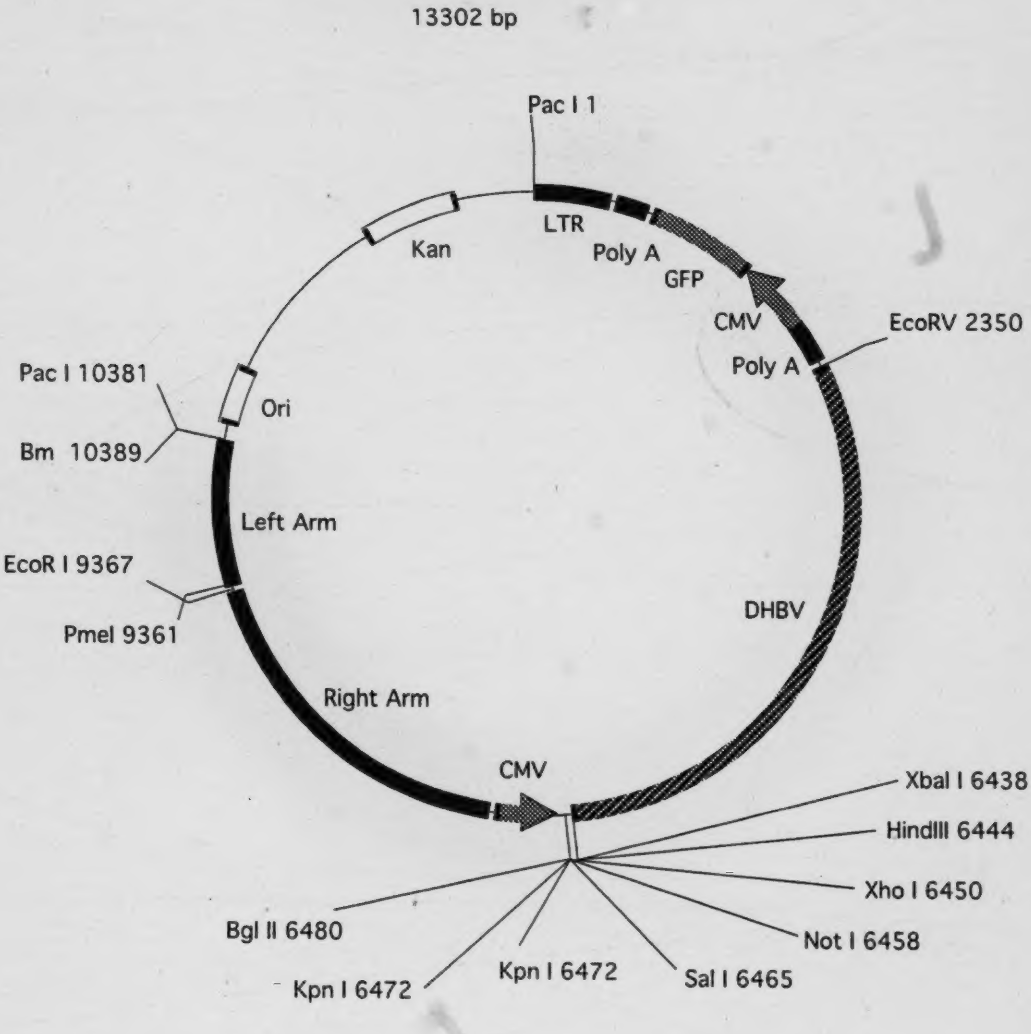
pAdTrack-CMV-DHBV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